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ADULT PLANT RESISTANCE TO FUNGAL PATHOGENS OF WHEAT

JOHN ALLAN HOWIE

**A thesis submitted to the Open University
for the degree of Doctor of Philosophy**

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Sponsoring Establishment:

Plant Breeding International (Cambridge) Ltd.

Maris Lane

Trumpington

Cambridge CB2 2LQ

Collaborative Establishment:

John Innes Centre

Colney Lane

Norwich NR4 7UH

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ABSTRACT

There is evidence that wheat, as well as containing genes for resistance, also contains genes that promote susceptibility to disease. Mutagenesis of the variety Hobbit 'sib' produced a number of lines with increased resistance to yellow rust (Worland & Law, 1991). The aim of this research was to characterise the resistance of the Hobbit 'sib' mutants, to identify and map the mutations and to determine which conferred resistance. The incidence of genes promoting susceptibility in other varieties also formed part of the investigation since manipulation of these genes may enable the resistance of commercial varieties to be improved.

Significantly improved resistance to yellow rust relative to Hobbit 'sib' was demonstrated in 17 of the 21 mutants investigated, confirming the previous findings of Worland & Law, 1991 (IAEA Vienna, Mutation Breeding Newsletter 38: pp 2 - 5). Furthermore, resistance to brown rust and mildew was also demonstrated in a subset of mutants, with one mutant, I3-48, exhibiting high levels of resistance to all three diseases. The broad spectrum of resistance in I3-48 suggests that mutation has inactivated either a single gene for susceptibility with a common effect on all three diseases, or a number of genes for susceptibility, each specific for a different disease. No mutants were identified with significantly improved resistance to *Stagonospora nodorum* or *Septoria tritici*, suggesting that this method of inducing resistance may only be of value for obligate pathogens.

Using molecular techniques, deletions were identified in 12 mutant lines, and the deletions were spread throughout the genome. Several mutant lines contained more than one deletion. "Representational Difference Analysis" was used successfully to identify deletions in selected mutants and, in contrast to using RFLP probes, provided a more targeted approach to identifying deletions irrespective of chromosome location. Analysis of the segregation of deletion markers in F2 populations segregating for disease resistance identified a deletion on 4DL of I3-48 that segregated with yellow rust and brown rust resistance. This was due to linkage in the case of brown rust resistance, but the yellow rust resistance may be due directly to the deletion, possibly as a consequence of the removal of a gene for susceptibility to this disease. A number of deletions were identified, both in I3-48 and other mutants, that did not segregate with resistance. Therefore, the deletion markers may be useful both as tags facilitating the selection of deletions conferring resistance, and also for the removal of deletions not implicated in improving resistance.

The distribution of genes for susceptibility in several varieties, most of which are in commercial use, was examined using either segregational tests for allelism or backcross reciprocal monosomic analysis in order to identify targets for disease resistance improvement. Both these approaches proved to be unsuccessful. The reasons for this failure are presented and possible solutions described.

The nature and possible roles of genes for susceptibility are discussed.

ABBREVIATIONS

Adw	Hobbit 'sib' monosomic
Bdw	Hobbit 'sib' monosomic
bp	base pairs
Bez	Bezostaya
cm	centimetre
cM	centimorgan
cpm	counts per minute
CS	Chinese Spring
cv	cultivar
°C	degrees Celsius
DNA	deoxyribose nucleic acid
dNTP	deoxynucleotide triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
g	gram
x g	acceleration due to gravity
kb	kilobase pairs
l	litre
M	molar
mg	milligram
min	minute
ml	millilitre

mM	millimolar
mm	millimetre
μg	microgram
μg	microlitre
ng	nanogram
nM	nanomolar
O.D	optical density
PCR	polymerase chain reaction
pH	-log[H⁺]
RFLP	restriction fragment length polymorphism
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
SSC	standard saline citrate
s	second
T_m	Temperature at which 50% of an oligonucleotide has hybridised to the complementary sequence
U	units
UV	ultraviolet light
V	volts
w/v	weight to volume

CONTENTS

ACKNOWLEDGEMENTS	i
ABSTRACT	ii
ABBREVIATIONS	iii
CONTENTS	v
CHAPTER 1: GENERAL INTRODUCTION	1
1.1 Historical Significance of Wheat	2
1.1.1 Evolution of Wheat	3
1.2 The Impact of Plant Pathogens	6
1.2.1 Classification of Fungal Pathogens	7
1.2.2 Variation in Fungal Pathogens	8
1.3 Major Fungal Leaf and Ear Diseases of Wheat In Great Britain	10
1.3.1 Yellow Rust	10
1.3.2 Brown Rust	11
1.3.3 Powdery Mildew	12
1.3.4 'Septoria' leaf and ear diseases	13
1.3.5 Fusarium diseases	14
1.3.6 Eyespot	15
1.4 The Genetics of Resistance	16
1.4.1 Race Specific Resistance	16
1.4.2 Race non-specific resistance	18
1.4.3 Durable resistance	18
1.5 Molecular Markers for Disease Resistance	20

1.5.1 Generation of Molecular Markers	21
1.5.2 Application of Molecular Markers	22
1.6 Suppression of Disease Resistance	23
1.6.1 Detection of Genes for Susceptibility	24
1.6.2 Role of the D genome in suppression of resistance	29
1.6.3 Role of the A and B Genomes in Suppression of Resistance	32
1.6.4 'Genes for Susceptibility versus 'Suppressors - revisited	33
1.6.5 Null Alleles of Suppressors of Resistance	34
1.6.6 Mutation - a means of attaining null alleles of suppressors	36
1.6.7 Hobbit 'sib' disease resistant mutants	39
1.7 Project Objectives	42
 CHAPTER 2: MATERIALS AND METHODS	 45
2.1 Plant Materials	46
2.1.1 Variety 'Hobbit sib'	46
2.1.2 Hobbit 'sib' Deletion Mutants	46
2.1.3 Hobbit 'sib' Monosomic Series	47
2.1.4 Hobbit 'sib'/Bezostaya Single Chromosome Substitution Lines	48
2.1.5 Chinese Spring Nullisomic-Tetrasomic Stocks	49
2.1.6 Backcross Reciprocal Monosomics	50
2.1.7 Growing of Plants for Crossing	50
2.1.8 Crossing Procedure	51
2.1.9 Cytogenetic Techniques for the Examination of Mitotic	

Chromosomes	51
2.2 Molecular Biology Techniques	52
2.2.1 Large Scale DNA Extraction	52
2.2.2 Small scale DNA extraction	53
2.2.3 Quantitation of Genomic DNA	54
2.2.4 Restriction Enzyme Digestion of Genomic DNA	54
2.2.5 Electrophoresis of Genomic DNA for Southern Analysis	54
2.2.6 Southern Blotting	54
2.2.7 Prehybridisation	55
2.2.8 Probe Labelling	55
2.2.9 Hybridisation of Filters	56
2.2.10 Washing of Filters	56
2.2.11 Autoradiography	57
2.2.12 Generation of Cloned DNA for Radioactive labelling	57
2.2.13 Quantitation of Insert DNA	59
2.2.14 Representational Difference Analysis	59
2.2.15 Boiled Miniprep Plasmid Purification	62
2.3 Disease Inoculation	63
2.3.1 Yellow Rust Inoculation	63
2.3.2 Brown Rust Inoculation	64
2.3.3 Mildew Inoculation	64
2.3.4 <u>Stagonospora nodorum</u> Inoculation	64
2.3.5 <u>Septoria tritici</u> Inoculation	65

2.3.6 Rust Field Trials	65
2.3.7 <u>Septoria tritici</u> and <u>Stagonospora nodorum</u> Field Trials	65
2.3.8 Growth Chamber Tests	66
2.3.9 Glasshouse Test	66
2.4 Nomenclature relating to aneuploid stocks	66
2.5 Stock Solutions	67
 CHAPTER 3: THE DISEASE RESISTANCE OF HOBBIT 'SIB' MUTANT LINES	71
 3.1 Introduction	72
3.2 Pathogens	73
3.2.1 Yellow Rust	73
3.2.2 Brown Rust	74
3.2.3 Mildew	75
3.2.4 <u>Septoria tritici</u>	76
3.2.5 <u>Stagonospora nodorum</u>	76
3.3 The Hobbit 'sib' mutant lines	77
3.4 Disease Tests Layout	77
3.4.1 Yellow Rust Trials	77
3.4.2 Brown Rust Trials	78
3.4.3 <u>Septoria tritici</u> and <u>Stagonospora nodorum</u> Trials	78
3.4.4 Mildew Tests	78
3.5 Results and Discussion	79
3.5.1 Yellow Rust	79

3.5.2 Brown Rust	82
3.5.3 Mildew	83
3.5.4 <u>Stagonospora nodorum</u>	85
3.5.5 <u>Septoria tritici</u>	87
3.6 Summary	87

CHAPTER 4: MAPPING OF DELETED SEQUENCES IN

HOBBIT 'SIB' MUTANTS	90
4.1 Introduction	91
4.2 RFLP Screening	92
4.2.1 Chromosome Group 4	92
4.2.2 Group 5 Chromosomes	93
4.2.3 Group 7 Chromosomes	94
4.2.4 Other Chromosome Groups	94
4.3 Representational Difference Analysis	95
4.3.2 Screening of RDA Clones	97
4.3.2.1 Deletion Mutant Line I3-54	99
4.3.2.2 Deletion Mutant Line I3-27	101
4.4 Mapping of RDA Clones	102
4.5 Mutant Allelism	105
4.6 Discussion	108

CHAPTER 5: SEGREGATION ANALYSIS OF HOBBIT 'SIB'

MUTANT RESISTANCE	115
5.1 Introduction	116
5.1.1 Possible Segregation Scenarios	118
5.2 Materials & Methods	119
5.2.1 Segregating Populations	119
5.2.2 Disease Testing	122
5.2.3 Chi ² test for Independence	123
5.3 Results	123
5.3.1 Hobbit 'sib' x I3-27	124
5.3.2 Rialto x I3-32	124
5.3.3 Rialto x I3-48	126
5.3.4 Rialto x I3-54	130
5.4 Discussion	131
5.4.1 Recombination	132
5.4.2 The use of segregation analysis	133
5.4.3 Agreement with resistance location via monosomic analysis	134
5.4.4 Lower frequency transmission of deletions	136
5.4.5 Future work	136
5.4.6 Conclusions	137

CHAPTER 6: DISTRIBUTION OF GENES FOR SUSCEPTIBILITY

IN VARIETIES OTHER THAN HOBBIT 'SIB'	138
6.1 Introduction	139
6.1.1 Bezostaya substitution line allelism	140
6.1.2 Backcross Reciprocal Analysis of Commercial Varieties	142
6.2 Bezostaya/Hobbit 'sib' Mutant allelism	143
6.2.1 Disease testing	145
6.2.2 Results	146
6.2.3 Discussion	151
6.3 Backcross reciprocal monosomic analysis	154
6.3.2 Materials & Methods	155
6.3.3 Development of Reciprocal Monosomic Lines	156
6.3.4 Field Trials	162
6.3.5 Data Analysis	163
6.3.6 Results	164
6.3.7 Discussion	169
6.4 General Discussion	174
 CHAPTER 7: GENERAL DISCUSSION	175
 7. General Discussion	176
7.1 The induction of resistance via mutation	176
7.1.2 Resistance to the Septoria diseases	177
7.2 Mapping of deletions	178

7.3 Segregation of deletions with resistance	181
7.4 Distribution of genes for susceptibility amongst	
commercial varieties	185
7.5 The expression of mutation induced resistance	186
7.6 What purpose do genes for susceptibility serve?	188
 REFERENCES	 192

CHAPTER 1
GENERAL INTRODUCTION

1.1 Historical Significance of Wheat

It can be said that wheat, or rather its ancestors, is responsible for civilisation as we know it. Since man's first successful attempts, about 10,000 years ago, to produce food rather than simply gather it from his surroundings, the history of cultivated wheat and human civilisation has been closely inter-twined. Within this time, the wheat plant lost the ability to disseminate its seeds effectively, and became completely dependent upon man for its dispersal. The domestication of wheat led to communal settlements, population increase and rapid cultural development. Man became involved to such an extent that wheat is now the worlds foremost cereal crop.

After the retreat of the Pleistocene ice age (about 12,000 years ago), periods of fluctuating and progressively increasing desiccation in certain parts of Asia became associated with large diurnal and seasonal variations in temperatures. This favoured the evolution of the annual habit in plants as a means of escaping unfavourable seasonal environments. It is proposed (Whyte, 1977) that this move to the annual habit facilitated the domestication of cereals by primitive man. Coincident with the reduction in availability of food from the usual perennial sources, the increased availability of the large-grained annuals, adapted to completing their growing season in the short period available to them would have favoured their use as a food source.

The gradual domestication of crops led to a tendency for nomadic man to become more stationary, in order to protect crops and territory, leading to a gradual development of civilisation, manifesting itself in a more complex social structure and technological advances. As surpluses were produced, storage of reserves could act as insurance against adverse years when yields were low, thus sustaining urbanisation and enabling population increase. Indeed, the production of surpluses would lead to an increase in trading and communication. The ability of a few producers

to support a number of consumers would release others to undertake different tasks within the community, leading to a transformation of the quality of existence and a more complex cultural civilisation.

Whilst domestication of wheat and its subsequent production has had a very significant influence on the development of civilisation, from its initial domestication through to modern times, it is the 20th century that has seen a phenomenal expansion and advancement in world wheat production, and hence its influence upon society. World wheat production has increased from 82.6 million tonnes in 1900 to in excess of 590 million tonnes in the 1990s, with approximately 200 million tonnes of that increase occurring in the last 20 years. Wheat is the most widely cultivated food plant in the world (F.A.O, 1995). It is arguably the most important world food crop and also exceeds the combined contribution of all other cereals entering into continental trade. As such, it holds the dominant position in agriculture, nutrition and commerce in today's society.

1.1.1 Evolution of Wheat

The exact origins of the modern wheat plant are unknown. Wheat is thought to have originated in the Middle East, in an area known as the 'fertile crescent', now Iraq and Syria. The evolutionary stages of the main wheat species are summarised in Fig 1.1.

The earliest known carbonized grains of the brittle diploid wheat, *Triticum boeoticum*, occur in the prehistoric settlements in northern Syria, where the cereal was apparently collected rather than farmed (Feldman, 1976). At later sites, carbonised remains give evidence of a transition from brittle to non-brittle diploid *T. monococcum* wheats around the 6th - 7th millennium BC. During the 5th millennium, cultivated einkorn (*T. monococcum*) spread to central and western Europe through the Danube and Rhine valleys. In subsequent eras (Bronze and early

Iron Ages), it attained a wide distribution in Europe and the Near East.

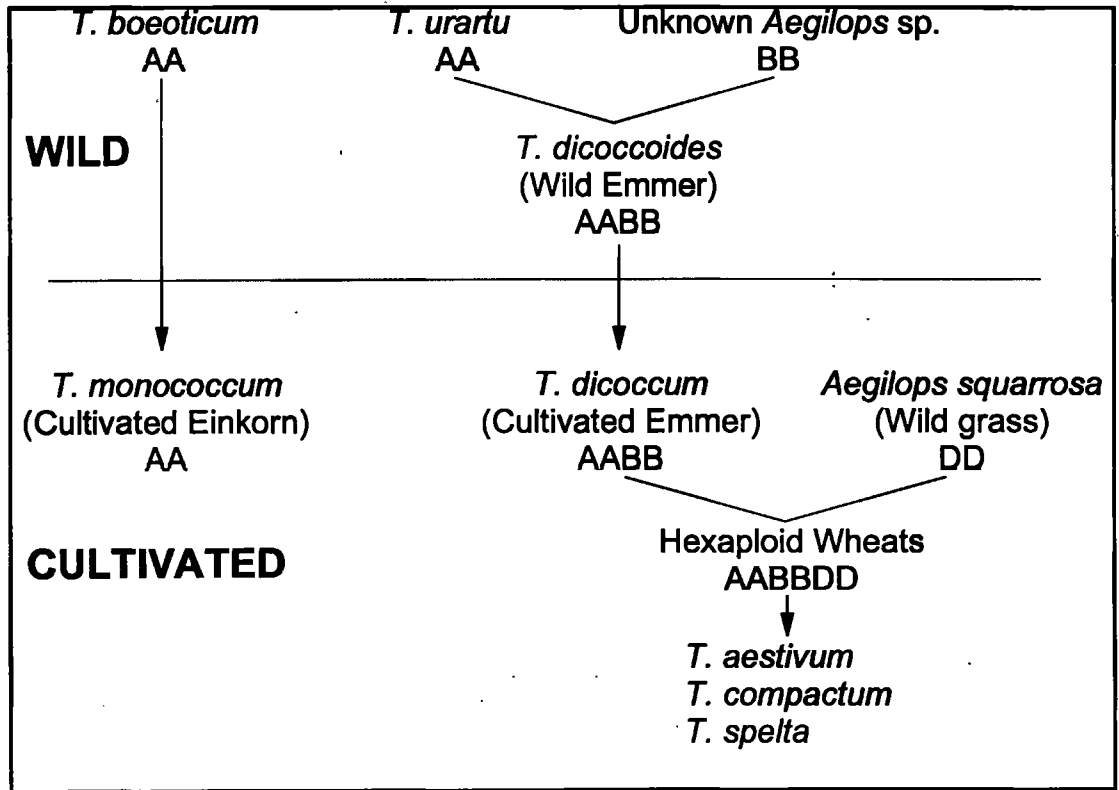


Fig 1.1: The evolution of the major polyploid wheats (after Miller, 1987)

Together with einkorn, the tetraploid cultivated emmer wheat (*T. dicoccum*), distinguishable by a non-brittle rachis, spread rapidly in the 7th millennium BC to farming areas in the Near East. It is thought to have been derived from an alternative ancestral A genome parent to einkorn (Miller, 1987). The initial step involved the evolution of the wild tetraploid, *T. diccoides*, by amphiploidy between a wild diploid *T. urartu* (AA) and an unknown diploid grass species similar to certain members of the Sitopsis group of *Aegilops* species. Cultivation of *T. diccoides* led to the evolution of *T. dicoccum* (cultivated Emmer). Emmer wheat moved to Egypt, the Mediterranean basin, Europe, Central Asia and India in the 5th and 4th millennium where it remained the principle cereal until the 1st millennium BC, when it was replaced by a

free-threshing form, namely durum wheat (*T. durum*). It is possible that durum wheat was actually derived from emmer wheat by a series of mutations that reduced the toughness of the glumes to a point at which the free-threshing state was achieved. It should be noted that durum wheat is just one of several tetraploid forms derived from Emmer.

Hexaploid wheats (AABBDD) are thought to have resulted from amphiploidy between the tetraploid *T. dicoccum* (AABB) and the D-genome donor *Aegilops squarrosa*. It is thought the D genome enabled the adaptation of hexaploid wheats (or bread wheats) to more continental climates. They appear in archaeological data from the 7th millennium BC, and are attributed to club wheat, *T. compactum*. Most early remains are found in Syria and Iraq. Remains of *T. aestivum*, dating from the 5th millennium BC, were found in the Nile basin as well as the central and western Mediterranean basin. Compact forms (*T. compactum*) were cultivated in central and western Europe until the end of the 4th millennium, where they are found associated, together with einkorn and emmer wheats, with the first traces of agricultural activities.

The main bread wheat, *T. aestivum*, was probably cultivated in the UK in about 2000BC, but it was not until 1529 AD that wheat was introduced to the New World, when the Spaniards took it to Mexico. Wheat finally reached Australia in 1788. *T. aestivum* is economically by far the most important hexaploid and is grown on a world wide scale. *T. aestivum* has been subject to extensive breeding effort, particularly in the 20th century, leading to vast yield, resistance and quality improvements as well as numerous other characters. The directed transfer of genetic material, rather than simple selection of pre-existing variability has enabled these advances. The identification and combination of a number of desirable traits from different parents, followed by rigorous selection for individuals containing that combination has enabled the advancement of wheat production. Increased world communication has also facilitated integration of genes

from various parts of the world and has enabled greater migration of desirable traits. Advancement has been due to not only pure breeding, but also the huge advances in the support disciplines i.e. research, that have enabled identification of a large number of these traits.

Modern wheat cultivars have therefore developed through three main phases of selection: subconscious selection by early growers; deliberate selection of variants in the field by the primitive farmer; scientifically planned modern breeding. The first phase would have been responsible for the selection of non-brittle spikes, simultaneous ripening and germination of grains and possibly free-threshing. Secondly, primitive farmers exerted various selection pressures, dependent upon their needs. Thus, increased yield, larger seed size, improved quality and adaptation to a wider range of climates and regimes would have been attained. Ultimately, modern breeding has enabled targeted improvement via relatively directed transfer of desired genes, with increased movement of useful traits around the world.

1.2 The Impact of Plant Pathogens

Plant pathogens have caused problems to human civilisation for centuries. This is clearly demonstrated by the inclusion in the Old Testament of blasting and mildew along with human disease and war, among the great scourges of mankind. The Romans became so painfully aware of the devastating effects of rusts of grain crops, they created a special rust God, Rubigo, to protect them.

Disease epidemics, unfortunately, are not phenomena restricted to ancient history. More recently, perhaps one of the most infamous examples of the effect of disease upon society was the outbreak of potato blight in Ireland in 1845 and 1846. The disease virtually wiped out the country's entire crop and, the resulting widespread famine led to a decline in population from

8.2 million in 1841 to 6.2 million in 1851, due to either starvation or emigration to Britain and North America (Russell, 1978).

In the last three decades, losses due to disease epidemics in wheat have been huge. A severe brown rust epidemic in 1978 in Pakistan resulted in the estimated national loss of \$US 86 million (Hussain *et al.*, 1980). Estimated crop losses of up to 50% due to brown rust infection in Egypt have been reported (Abdel Hak *et al.*, 1980). In 1992, large losses were incurred due to a widespread brown rust epidemic in Western Australia. In susceptible varieties, yield losses of up to 37% occurred, with an average loss of 15% across many fields (McIntosh *et al.*, 1995). Losses in Europe are primarily associated with yellow rust and brown rust.

On a worldwide scale, it is estimated that fungal diseases are responsible for an annual reduction of more than 20% of total potential world food production. This can have a significant impact on man, particularly in cases of epidemics, both directly and indirectly in terms of world trade and financial markets. These large effects reflect the need for continued attention to research into crop protection to reduce epidemic levels.

1.2.1 Classification of Fungal Pathogens

Plant pathogens can be grouped into two main categories, according to their nutritional processes, namely obligate pathogens (biotrophs) or facultative pathogens (necrotrophs).

Obligate pathogens

Obligate pathogens parasitize only living host plant tissues. In the natural environment, they grow and reproduce only on the living host and are usually dependent upon the host for their nutrition. These pathogens usually obtain their nutrients through specialised organs, haustoria, which are pushed into cells of susceptible host plants. The cytoplasm of the pathogen and host do not come into direct contact, but are separated by a series of host and fungal membranes

through which nutrients are transported.

As most obligate fungal pathogens feed on living host cells through haustoria, it is likely to be to the advantage of the pathogen if infected cells are not quickly killed or severely damaged. Severe damage does not generally occur until sporulation. For example, rust sporulation causes rupture of the epidermis and cuticle so that uredospores can be released into the air.

Facultative or non-obligate pathogens

Facultative pathogens kill host cells before progressing through the host tissues. They can feed saprophytically and can survive in the absence of living host plants. They may kill the cells of the host plant by secreting substances which either dissolve cell walls or destroy the cytoplasm. Alternatively, secretion of toxins by the pathogen may cause cell death.

1.2.2 Variation in Fungal Pathogens

Individual genotypes of a pathogen can vary from each other in many characteristics; the most important of which in terms of plant resistance to disease is pathogenicity (the ability to cause disease). Variation in pathogenicity is described in terms of virulence (C.A.B, 1973).

Variation in races, including the evolution of new races of pathogen with the ability to attack new host plants or varieties, i.e. new virulence, can be achieved by four main processes, namely mutation in somatic cells, recombination of nuclear genes during sexual reproduction, somatic recombination of genes or by fusion of haploid nuclei in the parasexual cycle.

Somatic mutations

Whilst changes in virulence may well occur through sexual recombination, this is limited by the genetic content of the parents. In absolute terms, changes in gene content must involve mutation. Mutations occur spontaneously and, if they confer a selective advantage, are likely to become fixed in the population. Mutations affecting virulence can alter the host range of a

particular race of pathogen. In the absence of a sexual phase, such as in yellow rust (*Puccinia striiformis*), mutation is the major source of variation. In addition, as mutations may be involved in subsequent recombination and so alter pathogen host ranges, mutation may thus be regarded as the basis for much of the variation in all plant pathogens.

Sexual recombination

Whilst sexual recombination in fungal pathogens is not necessary for the formation of new races, recombination at meiosis does promote the development of new variants with new combinations of virulence genes and thus potentially a new host range of pathogenicity.

Somatic recombination

Some important fungal pathogens, such as the rusts, have heterokaryotic cells, which have two or more genetically different nuclei. They are formed by hyphal anastomosis between mycelia of two different parental genotypes. A particularly stable form, the dikaryon, exists in Basidiomycetes, including the rusts. The anastomoses of different hyphae from different pathogen races, followed by recombination in the fused somatic cell, can give rise to new races of pathogen (Little & Manners, 1969; Goddard, 1976).

Parasexual cycle

Fusion of haploid nuclei in heterokaryotic cells to give diploid nuclei, followed by mitotic recombination producing a random re-assortment of genetic material can give rise to new races of pathogen. In pathogens with no known sexual stage, mitotic recombination via the parasexual cycle is the only means of genetic reassortment and thus is very important in the evolution of new pathogen races.

1.3 Major Fungal Leaf and Ear Diseases of Wheat In Great Britain

Leaf and ear diseases, especially on parts of the wheat plant involved in the production of reserve materials, can diminish potential wheat yields to a considerable extent. There are seven major fungal pathogens that are of economic significance in Great Britain. Yellow rust, brown rust and mildew are all obligate pathogens, whilst *Septoria nodorum*, *Septoria tritici*, the fusarium diseases and eyespot are all facultative pathogens.

1.3.1 Yellow Rust

Yellow rust, or stripe rust, caused by *Puccinia striiformis*, is the most common of the rust diseases of wheat in the cooler, humid regions of cultivation. It is particularly important in North-west Europe (UK, Netherlands and Denmark), although it is widespread throughout the world.

Yellow rust is an autoecious rust distinguished by lesions, consisting of flecks or stripes (chains of small interveinal pustules). Pustules are 0.5 - 1mm in size and are bright yellow to orange. No sexual stage or alternate host is known, so it is dependent for its survival on the primary cereal host. The optimum temperature for spore germination and penetration is 8-12°C. Uredospores germinate on the leaf surface to form germ tubes which grow towards stomata. A penetration hypha enters the substomatal space where it forms a substomatal vesicle. Haustoria form in the adjoining mesophyll cells and the fungus spreads by intercellular mycelium and intracellular haustoria. The mycelium gives rise to spores which erupt as rusty pustules through the epidermis, forming characteristic yellow stripes. Uredospores are often followed by teliospores in the same pustule. Uredospores are primarily dispersed to re-infect the same host, although are likely to be responsible for inoculation of other hosts. Teliospores give rise to basidia and basidiospores, which can provide primary inoculation to re-infect the same host, or surrounding host plants.

In NW Europe, rapid disease development can occur in the spring with severe levels possible by May. Further cycles of infection may occur throughout the summer, although these will be curtailed by hot, dry weather. Although spore germination is environmentally sensitive, existing infections are less sensitive and colonisation of host tissues can proceed, even in high temperatures and from a single penetration event.

Yellow rust behaves as a strong sink, competing with the usual sinks in the plant. Infections in early plants weakens them, whilst later infection destroys foliage and enhances evaporation. Although potential crop losses can be high, actual losses in England and Wales are in the order of 0-2% nationally (Jones & Clifford, 1983).

1.3.2 Brown Rust

Brown rust, or leaf rust, caused by *Puccinia recondita*, is arguably the most important disease on a world wide basis. It is more important in warmer, humid and semi-humid regions. It can also be damaging in NW Europe, particularly the Netherlands and France, although it is not as important as yellow rust in these regions.

A heteroecious, macrocyclic rust, brown rust symptoms are identified by uredial pustules that are discrete, round to oblong and usually 2mm or less in length. Pustules are orange to brown in colour; the colour deepening with age. Pustules occur primarily on the upper (adaxial) surfaces of the leaf blades. The fungus persists mainly in the uredial stage, which can survive relatively low temperatures. Urediospore infections of autumn sown wheat survive as mycelial colonies within the leaf tissues. Spore germination occurs over a range of 5-25°C (optimum temperature 10-20°C). The life cycle is very similar to that of yellow rust, although the teliospores of brown rust provide primary inoculum for infection of the alternate host genera in the families Ranunculaceae, Boraginaceae and Crassulaceae.

Anticyclonic conditions are most favourable for disease epidemic development, as the dry, windy days favour spore dispersal whilst the cold nights and dew favour spore germination and penetration. Individual crop yield losses of 5-10% are common, but may be up to 40% on susceptible cultivars in epidemic years (Jones & Clifford, 1983). Losses in the UK are minimal and brown rust is certainly regarded as less important agronomically than yellow rust.

1.3.3 Powdery Mildew

Powdery mildew, caused by *Erysiphe graminis*, is a leaf and ear parasite occurring over the whole of Europe (infestation centre in NW Europe) in regions of high atmospheric pressure. The disease attacks the leaves, sheaths, stems and inflorescences of wheat. The stages of development are well defined. Conidium spores germinate with a primary germ tube. A secondary appressorial germ tube emerges that forms an appressorium below which a penetration peg attempts to breach the host cell wall. If this fails, secondary, tertiary and even quaternary lobes may be produced from the appressorium, each of which is capable of producing a penetration peg (Boyd *et al*, 1994). If an infection peg successfully breaches the cell wall, it swells to form a haustorium, through which the fungus derives nutrients from the plant. In a susceptible host, a superficial mycelium develops from which new haustoria are formed, and within 7 - 10 days begins to develop conidia, which can be disseminated by the wind.

When two mildew colonies grow closely together, a sexual generation may be formed. Cleistothecia are formed by anastomosis between hyphae and the nuclei fuse after a short dikaryotic phase. Haploid ascospores are formed in asci, which are actively discharged and dispersed by wind.

Disease intensity fluctuates from year to year depending upon environmental conditions. Development occurs readily between temperature ranges of 15-20°C, although a relative humidity

of 90% is required for conidial germination and infection. Liquid water will prevent germination, so long periods of rain will impede disease development, as will long periods of drought.

As an obligate pathogen, powdery mildew survives on volunteer cereals in the harvest period before infecting winter sown seedlings, overwintering as mycelium. The initial phase of epidemic development in March/April is a decisive time in terms of severity of the epidemic. Even under the most favourable conditions, development of one generation of this fungus takes 5-8 days. An economically significant epidemic only occurs when the disease is able to start early in the season.

1.3.4 'Septoria' leaf and ear diseases

Although commonly described as the 'Septoria diseases', the two pathogens do not belong to the same generic group. For the purpose of this thesis, glume blotch will be referred to as *Stagonospora nodorum* (anamorph), although the teleomorph is *Leptosphaeria nodorum* (Jones & Clifford, 1983). Leaf spot of wheat will be referred to as *Septoria tritici* (anamorph), although the teleomorph is *Mycosphaerella graminicola* (Jones & Clifford, 1983). On a world scale, *S. tritici* is of major importance. Whilst *S. nodorum* used to prevail in NW Europe, occurring in moist regions and in years of high precipitation, the last 20 years has seen a switch in predominance to *S. tritici*. In Europe, *S. tritici* is more important in regions adjacent to the North Sea and the Atlantic, but is spreading into more central regions of western Europe.

S. tritici symptoms can be identified initially as light green to yellow lesions on leaves. Lesions spread to form light brown, linear to irregular patches, in which prominent black pycnidia develop. There is often considerable defoliation as lesions merge.

Leaf lesions of *S. nodorum* can be up to 1cm long, yellow at first but becoming golden brown later. The shape of the lesion varies considerably, but is approximately elliptical,

surrounded by a darker margin. Lesions often coalesce, resulting in the death of the leaves. Light brown pycnidia form within diseases tissue and are usually less conspicuous than those of *S. tritici*.

Both diseases are found simultaneously on the plant and infect all above ground parts. There are distinct differences in the development of epidemics though. *S. tritici* occurs earlier, particularly in seedling populations. *S. nodorum* is more important at the adult plant stage. The development of both pathogens is dependent upon the environment; humidity being the most important factor. Development of the disease and evolution of epidemics can only effectively occur when the relative humidity is 98% or more (Shaner & Finney, 1976).

S. nodorum has lower humidity requirements. It originates on both infected seeds and via ascospores in crop debris, then multiplies on senescent leaves before moving to green tissue at higher inoculation levels. The disease spreads up the plant by splash dispersal. *S. tritici* usually originates in the crop debris and grass weed hosts, and has a more prominent effect on seedlings than *S. nodorum*. It moves onto and through the host with a similar dispersal method to *S. nodorum*.

1.3.5 Fusarium diseases

The Fusarium diseases are diseases principally of the stem base and head, exhibiting a variety of disease symptoms on a wide range of cereals and grasses. They have a widespread occurrence in the main cereal growing countries of the worlds, and cause significant reductions in yield and quality, especially in wheat. They can be associated with the seedling crop as well as the adult plant.

Mainly soil borne, they are true soil inhabitants possessing a highly competitive saprophytic ability. In initial attacks on germinating seedlings they will often cause pre-

emergence death, whilst plants surviving early attack may later succumb to foot and root rots, as well as ear diseases.

Base diseases are predominantly due to *Fusarium culmorum* and *Monographella nivalis* (*F. nivale*). Seedlings surviving initial attack may develop typical Fusarium foot-rot symptoms, which can vary from brown discolouration of basal leaf sheaths to more pronounced rotting of the plant base at soil level.

Ear diseases are caused mostly by *F. culmorum*, more common in cooler regions, and *F. graminearum* in warmer regions of central and southern Europe. Symptoms include poorly filled ears and shrivelled grains. There may also be a brown spotting on the glumes of infected plants and the whole ear may become tinted a red/pink colour. Toxins may also reduce grain quality. Infection of the ear is very dependent upon weather conditions at anthesis, being favoured by humid conditions. The infection 'window' is therefore small.

1.3.6 Eyespot

Caused by *Pseudocercospora herpotrichoides*, Eyespot is present in most temperate countries where it can cause considerable damage to cereals, mostly winter wheats and barleys. Symptoms are characteristic pale oval spots with a brown margin on the basal leaf sheaths and the culm of cereal tillers. The symptoms may develop in the spring and can be confirmed by the presence of grey mycelium in the straw cavity. The Eyespot pathogen can also result in the post emergence death of young seedlings or tillers; shrivelled grain and partially empty ears can occur on maturing crops. Foot rot of the stems can also cause 'lodging' of the crop.

The pathogen survives the inter-crop period on infected stubble or volunteer cereals. Conidia develop most abundantly during periods of wet autumn and spring weather. Incidence and development of the disease is much influenced by the environment, with infection being

severe at soil temperatures of 6-10°C. A heavy epidemic may result in losses of 5-10% in coastal areas of N/NW France, UK, Netherlands, Germany and S. Scandinavia.

1.4 The Genetics of Resistance

Resistance to pathogenic disease can be attributed to either resistance to the pathogen, disease escape or disease tolerance. Disease escape occurs when genetically susceptible plants do not become infected because the three factors necessary for disease (susceptible host, virulent pathogen and favourable environment) do not coincide sufficiently for disease to develop. This can be due to a number of factors including host characteristics and environmental variables.

Disease tolerance occurs when plants are infected to the same extent as other plants but are not damaged as much by that infection. Tolerant plants do not affect the development of the pathogen, which in turn has little effect on the host.

Each taxonomic group is host to a small and different set of pathogens that make up the total number of known plant pathogens, i.e. each group is non-host to the vast majority of plant pathogens. Non-host resistance is apparent when all genotypes of a species are resistant to the pathogen. Host resistance occurs when only some genotypes are resistant, or a varying level of resistance is apparent. Host resistance can be split into two categories: race specific and race non-specific. These categories have previously been described as vertical and horizontal resistance respectively (Vanderplank, 1963).

1.4.1 Race Specific Resistance

Resistance of the host to some races of a pathogen but not to others is described as race specific resistance. It is usually controlled by one or a few genes, referred to as monogenic or oligogenic resistance respectively.

Infection by an incompatible race of the pathogen frequently results in a hypersensitive reaction, involving restricted cell death and deposition of defence metabolites at the point of pathogen attack, preventing or restricting establishment and multiplication in the host.

Gene-for-gene specificity

The specificity of race-specific resistance suggests there is a relationship between the races of the pathogen and the host genotypes. This situation was first described by Flor (1956), working with flax rust fungus (*Melampsora lini*), as the 'gene-for-gene' concept. The plant-pathogen interaction was accounted for in terms of gene-for-gene complementarity, where specific matching gene pairs of host resistance (R) and pathogen avirulence (Avr) controlled the outcome between different combinations of host and parasite genotypes. It has become apparent that this is true for many host-parasite relationships (Crute, 1985). Essentially, unless an allele for host resistance is specifically matched by an allele for pathogen avirulence, compatibility will occur, involving extensive pathogen development and reproduction. Gene pairs resulting in incompatibility (R-Avr) are dominant over all other gene pairs that result in compatibility. This is summarised by the gene-for-gene relationship for 2 interacting pairs shown in Fig. 1.2.

Pathogen Alleles	Host Alleles			
	R1R2	R1r2	r1R2	r1r2
Av1,Av2	I	I	I	C
Av1,v2	I	I	C	C
v1,Av2	I	C	I	C
v1,v2	C	C	C	C

I = incompatible reaction C = compatible reaction

Figure 1.2:A gene-for-gene interaction between two host loci for reaction each with two alleles (R1 or r1, R2 or r2) and the corresponding pathogen loci for pathogenicity (Av1 or v1, Av2 or v2), after Johnson (1992)

1.4.2 Race non-specific resistance

Some plants have a certain, but not always the same, level of non-specific resistance, effective against all races of a pathogen and sometimes described as horizontal resistance. It should be noted that, in real terms, the resistance can only be classified as "apparently race non-specific" as a race may exist that has a specific effect on the host, but the host has not been exposed to that race.

Race non-specific resistance is commonly, but not necessarily, controlled by many genes and is often described as polygenic. Generally, this resistance does not protect the plant from becoming infected, but slows down the development of individual infection loci on the plant and therefore slows the spread of the disease. It can therefore be described as quantitative in effect, rather than the qualitative nature of race specific resistance.

1.4.3 Durable resistance

The longevity of disease resistance is of interest, particularly to plant breeders. The introduction of new resistances into a variety is often matched by previously unknown pathogen races, negating, at least in part, the effectiveness of that resistance. The resistance is described as having 'broken down', although this is a somewhat mis-leading term, as the resistance is still present, but has been matched by pathogen virulence. Certain resistance genes have remained effective for long periods of use and, providing they fit certain criteria, may be described as durable. Johnson (1984) described durable resistance as "resistance that remains effective during its prolonged and widespread use in an environment favourable to disease". As such, the term can only be used retrospectively. Nevertheless, the identification and understanding of the genetic basis of durable resistance can have important implications if durability in future breeding programmes is to be achieved.

Confusion over the genetic basis of resistance, and unwarranted assumptions about particular genetic controls has often lead to the incorrect identification of sources of durable resistance. It is often implied or assumed that race non-specific resistance is under polygenic control, and therefore is less likely to be overcome and may be durable. Whilst this may be so in many cases of durability, the converse is not always true. There are numerous examples of durable resistance due to single genes. A relatively recent and well known example of durable resistance being associated with a single gene is that of the role of *Lr34*, which confers durable brown rust resistance in wheat, as discussed by McIntosh (1992). Most durable resistance to brown rust has probably been due to combinations of *Lr34* with other brown rust genes, such as the cultivar 'Era' which has been resistant to brown rust since its release in 1971 and contains *Lr13*, *Lr10* and *Lr 34* (Ezzahari & Roelfs, 1989). German & Kolmer (1992) have examined paired combinations of brown rust resistance genes with *Lr34* in a common cv 'Thatcher' background. They found that in all tests, *Lr34* interacted for enhanced resistance. The authors did note, however, that not all enhanced combinations will be durable, although certain combinations such as *Lr34+Lr13* (the basis of the 'Era' resistance) are more likely to be (Roelfs, 1988).

Parlevliet (1988) predicted that the combination of several major genes, known commonly as 'gene stacking' would result in resistance that would be highly durable. He believed that the combination of several genes would be much harder for pathogens to evolve virulence to. This does rely on none of the genes being deployed singly, as this would enable the pathogen to evolve virulence in a stepwise manner. In breeding terms, this is very hard to achieve. It requires a knowledge of all the resistance genes involved, as well as an agreement between all breeders in the epidemiological region not to split the combination. Selection of the 'combined' genotypes is also very difficult, and requires biochemical or molecular markers to 'tag' the genes being

stacked. The resistance genes must confer resistance to all existing races. Therefore one can not distinguish between lines carrying the separate resistance genes or the combined genotypes. As it would be ideal to have a number of genes stacked, this makes the breeding process even more difficult.

The variability in genetic control of durability highlights not only the importance of not making assumptions regarding its control or the nature of the resistance, but also the difficulty in achieving resistance. Whilst components of durability may be identified, the true test of durability, i.e. prolonged and widespread usage, mean that a long timescale is required to demonstrate the effective introduction of those components into a variety.

1.5 Molecular Markers for Disease Resistance

The development of comprehensive genetic maps in plant species enables a greater understanding of the relationship between chromosome structure and gene function. These maps also have a potential impact on crop improvement as they provide plant breeders with the opportunity for the direct manipulation of genes, via 'marker-mediated' selection. Markers can be used in two ways. First, if the marker allele confers a desirable trait, it can be selected directly. Secondly, where a marker is phenotypically neutral, but is tightly linked to an agronomic trait of interest, the neutral marker can be used to select for the desired trait. An example of this is the linkage of an endopeptidase enzyme to eyespot resistance in wheat (Worland *et al*, 1988). However, the use of morphological and isozyme markers does not enable the realisation of the full potential of genetic mapping, as the number of these markers is relatively small, and morphological markers can cause such large effects on phenotype that they are undesirable in breeding programs and may mask the effects of linked genes.

1.5.1 Generation of Molecular Markers

The use of DNA polymorphism has allowed a much greater number of loci to be identified than could previously be marked by isozyme or morphological markers. This should enable the accurate location and manipulation of more agronomically important genes. These polymorphisms are due to base sequence changes or DNA rearrangements, and are naturally occurring, simply inherited, Mendelian characters.

Genetic maps have been constructed using restriction fragment length polymorphism (RFLP) analysis. Restriction endonuclease enzymes cleave genomic DNA into differing size fragments. The technique exploits variations on DNA sequence that create or abolish sites for restriction endonuclease cleavage, or which involve addition or deletion of DNA segments between sites. Methylation of the cytosine residues within the enzyme recognition site may also prevent restriction by certain endonucleases. Consequently, such enzymes may detect RFLP if there is variation in methylation patterns. RFLP can be visualised because the DNA from different genotypes is cleaved in fragments of different sizes, and these can be separated electrophoretically. RFLP analysis uses DNA sequences (probes) that are cloned from, or homologous to, DNA from the organism of interest. Hybridisation of the sequence specific probes to the RFLP allows detection of polymorphism. Hence, the DNA probes can act as markers for a DNA polymorphism, that may be associated with a trait of interest.

An alternative approach is to analyse RAPDs (random amplified polymorphic markers). Short synthetic oligonucleotides are used as primers in PCR (Polymerase Chain Reaction), with genomic DNA as a template (Williams *et al* 1990). The products of the PCR are separated electrophoretically, and polymorphism is revealed when DNA is either amplified from only one parent, or where DNA fragments of differing size are generated.

RFLPs and RAPDs have major advantages over conventional markers. Conventional markers rely on differences between gene products, which arise from alterations in gene coding or expression. In contrast, molecular markers directly reflect variation in the DNA. Thus, the number of potential sites of DNA polymorphism is much greater than the number of conventional markers. RFLPs will also enable the distinction of both dominant and recessive alleles. DNA markers also offer entry points in the genome, from which, in theory, any gene can be isolated.

A number of marker systems have now been developed in addition to RFLP and RAPD marker systems. These include microsatellites, amplified fragment length polymorphisms (AFLP) and retrotransposons. A review of these systems can be found in Powell *et al* (1996).

1.5.2 Application of Molecular Markers

Location of Resistance Genes

The utility of molecular markers for studying disease resistance is based on finding close linkages between markers and the genes for resistance. Such linkage enables the presence of a desirable gene to be inferred by assaying for the marker. Incorporation of resistance genes into varieties requires crosses with stocks that carry the resistance gene, followed by selection among the progeny for individuals possessing the desired gene combination. This is traditionally done by screening for resistance to the pathogen race in inoculation tests. Difficulties can be experienced with this procedure, particularly if there are a number of pathogen races to screen. Detection of resistance genes by their linkage to molecular markers enables the screening of several disease resistance genes, without the need to inoculate. This obviates any environmental effects that may influence phenotypic selection, and could be of particular relevance for stacking of resistance genes, where it may be desirable not to expose resistance genes to pathogen inoculation until the genes are grouped, reducing the risk of evolution of pathogen virulence to

the gene combination.

Molecular markers for disease resistance have been identified in a number of crop species. For example, RFLP and RAPD markers tightly linked to downy mildew resistance have been isolated in lettuce (Paran *et al*, 1991). Polymorphisms were detected by screening near isogenic lines differing for the resistance gene *Dm11*, a number of which were tightly linked to the resistance gene. A number of resistance genes have also been mapped in cereals, such as the *Rpg1* locus for resistance to stem rust resistance in barley (Killian *et al*, 1994) and *Lr 10* in wheat (Schachermayr *et al*, 1997).

Cloning of resistance genes

Mapping of resistance genes also provides entry points from which resistance genes can be cloned. Cloning enables the study of the structure and function of the genes which, once cloned, could be used for transformation. Molecular markers are important for cloning when the products of the genes are unknown and therefore can not be used as a means of 'targeting' the gene. RFLP markers are physically linked to the gene and therefore can be used as a starting point from which the gene can be isolated. This has been well demonstrated in the cloning of the *Mlo* gene in barley (Buschges *et al*, 1997), where markers tightly linked to the *Mlo* locus have enabled physical isolation of the locus and cloning of the gene.

1.6 Suppression of Disease Resistance

Adult plant resistance genes (conferring disease resistance to the adult plant) have been extensively studied and documented (McIntosh *et al*, 1995) and these have been used extensively in breeding programmes to achieve improved adult resistance. Adult plant resistance genes are present in a number of wheat species and their relatives. Transfer of resistance from a number of

relatives into wheat has been achieved (Kerber & Dyck, 1973). However, not all transfers of resistance were successful (The & Baker, 1975). A number of examples exist, to be discussed in detail later, where resistance is not expressed in the new genetic background, resulting in a lack of resistance or even increased disease susceptibility. In other cases, aneuploid analysis of varieties has indicated that genetic factors are present within the varieties that increase the susceptibility of the variety or alternatively reduce or suppress resistance.

Dependent upon the author, these factors may be described as genes for susceptibility (Law *et al*, 1978; Pink *et al* 1983), or suppressors of resistance (Kerber & Green, 1980; Multani *et al*, 1989; Kema *et al*, 1995). Which ever definition is used, the end result is the same; susceptibility of the variety. The difference between the definitions is that the term 'gene for susceptibility' describes the effect, whereas 'suppressor of resistance' also implies function. For the purpose of the description of the work contributing to this field in the following section, the authors' definitions shall be used in each case. For the remainder of the thesis, the term 'genes for susceptibility' will be used. The work forming the foundation on which the majority of the research detailed in this thesis was based refers to 'gene for susceptibility'.

1.6.1 Detection of Genes for Susceptibility

Aneuploid lines of the varieties Bersee and Cappelle-Desprez were used to analyse the effects of the chromosome dosage on yellow rust resistance in the two varieties (Johnson & Law, 1975; Law *et al*, 1978). Using monosomic series for both varieties, they found that chromosome 5BS-7BS played an important role in determining resistance in both varieties, illustrated by an increase in disease level in the monosomic. With the Cappelle-Desprez series, the moderate level of disease susceptibility in the euploid also enabled the detection of a number of monosomic lines with increased resistance relative to the euploid control. Most notable of these were 5A and 5D,

both of which showed a highly significant increase in resistance in the monosomic state. In addition, 4D and 2D showed an increase in resistance relative to the euploid. This suggests that these chromosomes carry genes that increase susceptibility in the euploid. Reduction in dosage of these genes in the monosomic leads to a reduction in disease level. Tetrasomic 5A, 5B and 5D of Chinese Spring were also investigated. The increased dosage of each chromosome resulted in an increase in disease level, with 5A giving the largest increase in disease level. Investigation of the nullisomic 5A tetrasomic 5B gave a reduction in susceptibility. In addition, substitution of 5A with 5R from rye also reduced the disease level. This suggests that 5A carries a gene for susceptibility, that increases disease susceptibility at increased dosage, whilst removal of this gene either by substitution of the chromosome or by aneuploidy results in reduced dosage and thus reduced disease levels. A model to describe the effect of chromosome dosage on disease levels when genes for susceptibility are involved is shown in Fig 1.3.

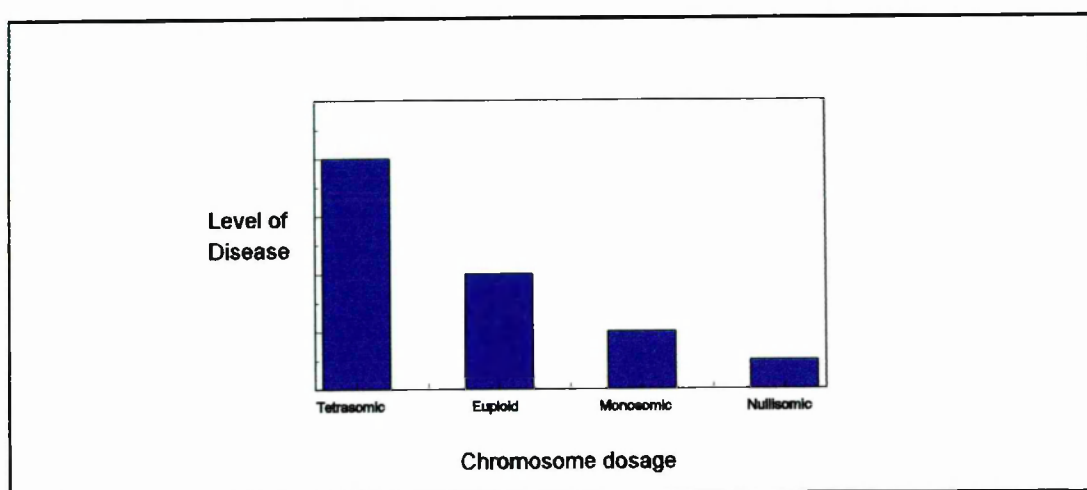


Figure 1.3: Effect of chromosome dosage on genes promoting susceptibility

Work on the Chinese Spring group 5 chromosomes was extended further by Pink *et al* (1983). Using tetraploid and monosomic lines, the effect of chromosome dosage on resistance to yellow rust and mildew was examined. As with the results of Law *et al* (loc.cit), there was a

significant increase in infection with increasing dosage in each of the group 5 tetrasomics when tested with yellow rust, and a reduction in infection in the monosomic lines. It was noted that 5D had less of an effect than 5A or 5B. There was also a correlated effect for mildew in both the tetrasomic and monosomic lines. Lines ditelosomic for the long arms of the group 5 chromosomes were also studied. Removal of the short arms of each chromosome resulted in a significant increase in infection of the leaves of the Chinese Spring ditelosomic lines for both yellow rust and mildew, indicating that the short arms must therefore carry genes contributing to resistance in the euploid. However, reduction of chromosome dosage in the monosomic lines resulted in a decrease in infection. It was therefore concluded that the long arms of the group 5 chromosomes must carry genes for susceptibility to yellow rust and mildew, whose effects are greater than those of the genes for resistance on the short arms. It was proposed by the authors that, as the effects were correlated for two taxonomically unrelated pathogens, the loci may influence the basic physiological relationship between wheat and its foliar pathogens.

The genetic control of adult plant resistance to yellow rust in the susceptible variety Hobbit 'sib' has been investigated using a monosomic series in the variety (Worland & Law, 1991). The results showed that eight chromosomes carried genes for resistance, demonstrated by an increase in disease infection in the monosomic line (Figure 1.4). Chromosomes 1A, 2A, 2B, 2D, 4A, 5BS-7BS and 6D all showed significant susceptible deviations from the euploid control. This suggests that Hobbit 'sib', although susceptible, still carried genes for background resistance on the above 7 chromosomes, without which levels of infection on the euploid would be higher. Five chromosomes gave lower infection levels than Hobbit 'sib' when in the monosomic state, suggesting the presence of genes for susceptibility on 3B, 4B, 4D, 5BL/7BL and 5D. Additional scoring of ditelocentric 7BL, lacking the long arm of 5B, indicated reduced levels of infection

associated with monosomic 5BL-7BL were primarily due to the loss of 5BL. This is in agreement with the work of Pink *et al* (1983), where it was shown that Chinese Spring 5BS carried a gene for resistance, whilst 5BL carried a gene for susceptibility.

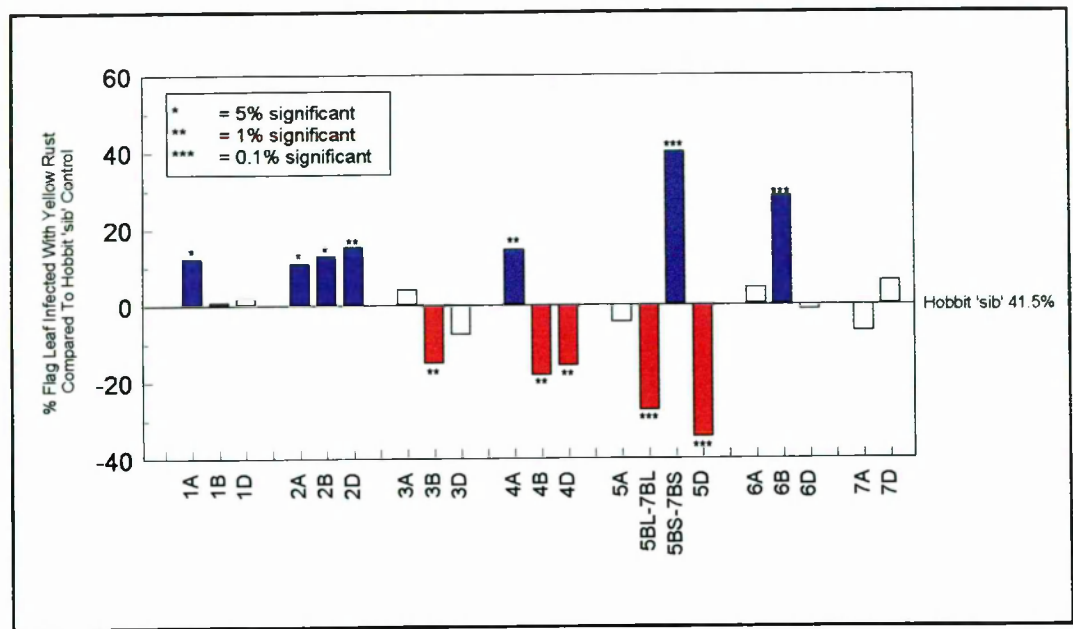


Fig 1.4: Monosomic analysis of Yellow Rust Infection in Hobbit 'sib'. The bars represent the deviation of flag leaf infection level from Hobbit 'sib for each of the monosomic lines.

It was proposed by Worland & Law (1991; see also Law *et al*, 1978) that improved levels of resistance could be achieved by replacing chromosomes carrying genes promoting susceptibility by homoeologous chromosomes from other varieties carrying less potent genes for this character. This was tested by analysing Hobbit 'sib'/Bezostaya single chromosome substitution lines, as illustrated in Fig. 1.5.

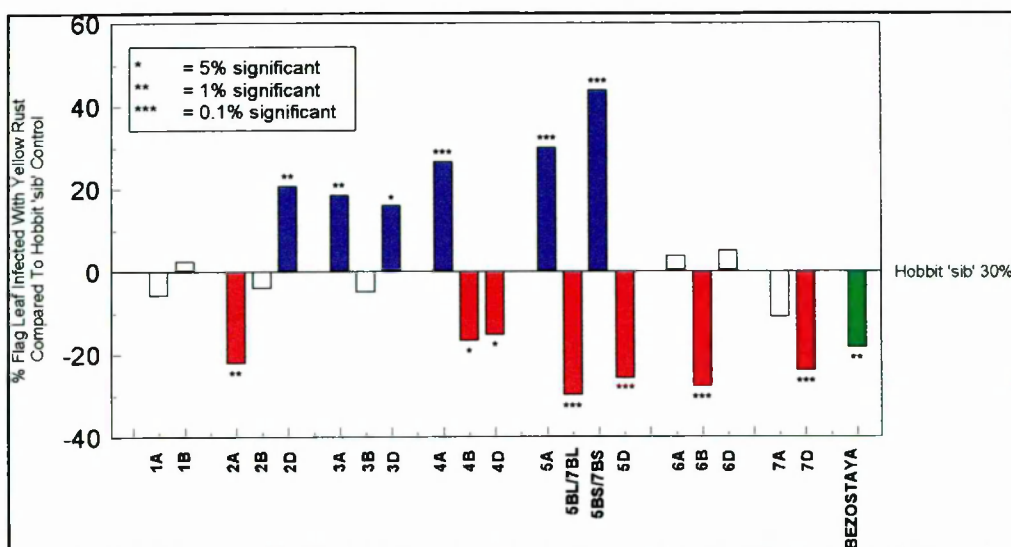


Fig 1.5 Relative flag leaf infection of Hobbit 'sib'/Bezostaya substitution lines. The bars represent the deviation of flag leaf infection from Hobbit 'sib' for each of the substitution lines and the variety Bezostaya.

Bezostaya has a lower level of disease infection than Hobbit 'sib' in the euploid state. Analysis of the 20 available substitution lines showed that 13 lines deviated significantly from the recipient varietal control. Where the Bezostaya substitution line was more susceptible than the euploid, it was assumed that the Bezostaya chromosome was less efficient at promoting disease resistance than its Hobbit 'sib' homologue, either because it carried weaker alleles for the promotion of resistance, or stronger alleles for the promotion of susceptibility. However, seven Bezostaya chromosomes were more efficient at promoting disease resistance, including 2A, 4B, 4D, 5BL-7BL, 5D, 6B and 7D. Four of these chromosomes were previously highlighted by Worland & Law (1991) as promoting susceptibility in Hobbit 'sib', indicating alleles for susceptibility on these chromosomes are less potent in Bezostaya than in Hobbit 'sib'. Alternatively, the Bezostaya chromosome may carry alleles promoting resistance. It is known to possess Lr34/Yr18 and may possess Yr2 (R. Johnson, pers.comm).

1.6.2 Role of the D genome in suppression of resistance

Aneuploid studies were used by Kerber & Green (1980) to determine stem rust resistance in the variety Canthatch. As with the work of Law *et al* (1978), it was noted that reduction in dosage of certain chromosomes resulted in increased resistance, again suggesting the presence of genes for susceptibility, or 'suppressors of resistance', as described by the authors. In addition, the tetraploid Tetracanthatch (AABB) was resistant whilst Canthatch was susceptible, indicating the D genome determined susceptibility. Canthatch nullisomic 7D was significantly more resistant at the seedling stage than the euploid for a number of races of stem rust, although race-specificity was noted. Testing of ditelosomic Canthatch 7DL gave disease levels similar to the euploid variety, indicating 7DS has no effect on disease resistance and that the susceptibility loci mapped to 7DL. At the adult stage, the results of the seedling investigations were generally confirmed, but with more pronounced race-specific effects. Substitution of 7D from *Aegilops squarossa*, the proposed donor of the D-genome in bread wheats, into Canthatch resulted in similar disease levels to Canthatch, suggesting the 'suppressor of resistance' was present in the ancestral progenitor as well the modern variety. It was proposed that the introduction of the suppressor(s) of resistance from the D-genome into bread wheat resulted in suppression of genes determining resistance found on the A and B genomes.

Further work on chromosome 7D by Kerber (1983) tested the 7D chromosome of six varieties. Chromosome 7D from each of the varieties was substituted into Canthatch and the F1 generations were tested for stem rust resistance. Synthetic hexaploids with *Triticum tauschii* were also made and used to produce *T. tauschii* 7D substitution lines in Canthatch. All the substitution lines were susceptible to stem rust, suggesting that all the 7D sources contain the 7D suppressor identified previously. These results further support the hypothesis that the suppressor was derived

from the ancestral genome. It should be noted that no 7D effect was detected by Worland & Law (1991) in their work on adult plant resistance to yellow rust in Hobbit 'sib'. Interestingly, the suppression of resistance in Canthatch is race-specific, whereas the adult plant resistance in Hobbit 'sib' is apparently race non-specific. This suggests that the suppression determined by 7D in Canthatch is specific either to resistance genes in Canthatch, or alternatively is disease specific, and is effective only against stem rust.

Additional evidence of the role of 7D in the suppression of resistance comes from work by Zeller & Hsam (1996) investigating suppression of the dominant mildew resistance genes *Pm8* and *Pm17*. Two cultivars, Disponent (*Pm8*) and Helami-105(*Pm17*) were crossed onto the cultivar Caribo. All F1 progeny were susceptible, with some resistant progeny segregating out at F2. This suggests that Caribo carries a dominant suppressor of both *Pm8* and *Pm17*. Further crosses were made using the Caribo monosomic series as the female parent. The F1 monosomic hybrids from all crosses were susceptible, except for those involving 7D. The 7D progeny, where the 7D chromosome was always derived from the resistant male parent were all resistant. This indicates that the suppressor of both mildew resistance genes is located on chromosome 7D.

The general involvement of the D genome in suppression has been noted by a number of workers. Kerber & Green (1980) noted problems transferring stem rust resistance from diploid (AA) and tetraploid (AABB) wheats into hexaploids. It was suggested that the D genome was suppressing these resistance genes upon introduction. Bai & Knott (1992) have demonstrated the presence of suppressors of stem and brown rust race-specific resistance on the D genome. Resistant wild tetraploids were crossed onto both durum and bread wheats. The resistance was expressed in the durum background but not the hexaploid background, suggesting an inhibitory effect of the D genome. Furthermore, the crossing of resistant durum lines with susceptible

hexaploid lines led to the suppression of the durum resistance in the hexaploid background.

Investigation by Bai & Knott (1992) of seven Chinese Spring monosomic lines, monosomic for the D chromosomes, by crossing to three resistant dicoccoides accessions demonstrated the presence of putative suppressors on several of the D chromosomes. Chinese Spring 1D, 2D and 4D were all shown to suppress stem rust resistance in all three dicoccoides accessions. Chromosome 3D suppressed brown rust resistance in all three accessions, whilst 1D suppressed brown rust resistance in only one accession. This indicates that the suppressors were specific to certain chromosomes or resistance genes. It should be noted that tests of Chinese Spring 7D (Kerber, 1983) identified the chromosome to carry the 'Canthatch' suppressor. This was not indicated by these studies, using different stem rust races, suggesting that suppression was either race-specific, or alternatively resistance gene specific.

Evidence for specificity of resistance gene suppression has also been obtained for crown rust (*Puccinia coronata*) resistance in oat. Chong & Aung (1996) investigated the interaction of the crown rust resistance gene *Pc94* with five other crown rust resistance genes by crossing the *Pc94* line to five single resistance gene lines. F1 progeny from all five crosses were tested for resistance to isolates virulent to the five single *Pc* genes but avirulent to *Pc94*. Four of the families were resistant, suggesting *Pc94* to be dominant. However, progeny of the *Pc94/Pc38* cross were susceptible, suggesting that either *Pc94* was recessive in the cross, or its expression had been suppressed. Analysis of the F3 segregation of *Pc38/Pc94* with an isolate virulent to *Pc38* but avirulent to *Pc94* showed a ratio of 1:8:7 resistant:heterozygous:susceptible, rather than a 1:3 ratio expected for a recessive allele. This suggests that *Pc94* is specifically suppressed by *Pc38*, possibly as a result of inter-genomic competition when the two genomes containing *Pc94* and *Pc38* are brought together by crossing. A more general theory of intergenomic competition,

resulting in suppression of resistance, has been proposed by Aung (pers. comm). He proposes that the combination of genomes, as occurs in polyploid species such as wheat, results in inter-genomic competition and requires a certain level of gene silencing in order for successful polyploidy to occur. It is possible that certain resistance genes have been silenced, or suppressed, possibly by 'competing' resistance genes as a result of the combination of the A, B and D genomes in wheat.

Singh *et al* (1996) tested the expression of certain *T. durum* cultivar yellow rust resistance genes when crossed with different *T. tauschii* accessions (D genome donors). Only certain accessions suppressed resistance, again indicating a specificity of suppression.

1.6.3 Role of the A and B Genomes in Suppression of Resistance

As with earlier work, the role of the D genome in suppression of resistance has been demonstrated by Singh *et al* (loc. cit). However, the authors also demonstrated suppression by the A and B genomes, where the resistance of certain *T. tauschii* accessions was not expressed in the synthetic hexaploid. Suppression of *T. tauschii* resistance by tetra Canthatch (AABB) was also demonstrated by Kerber (1983), indicating the presence of suppressors on the A or B genomes. Suppression appeared to be race-specific, or more likely resistance gene specific. Race non-specific and disease non-specific suppression or susceptibility has been located to the B genome in the tetraploid cultivar 'Langdon' (Multani *et al*, 1989), using nulli-tetra and ditelosomic lines. The lines were inoculated with a 5 race yellow rust mix and a 6 race brown rust mix. Nullisomic 7B was resistant to both rusts, indicating that 7B carried a gene for susceptibility. In addition, ditelosomic 2BL and 7BL had low disease levels, indicating that genes for susceptibility were located on the short arms of 2B and 7B. This work parallels the work of Pink *et al* (1983), where the long arms of the group 5 chromosomes carry genes for susceptibility, and show

correlated effects between yellow rust and mildew. There are also similarities with the work of Worland & Law (1991; unpublished), where chromosomes 3B, 4B and 5BL-7BL, as well as 4D and 5D, in Hobbit 'sib' were identified to carry genes for susceptibility, confirming that suppression or susceptibility is not confined to the D genome.

Innes & Kerber (1994) noted the action of one or more intergenomic suppressor loci on the AB genome. Analysis of 12 *T. tauschii* accessions enabled the identification of a number of seedling and adult brown rust and stem rust resistance genes. However, when crossed with Tetracanthatch to form synthetic hexaploids, 2 of the four seedling brown rust resistance genes were not expressed. Further evidence for the presence of suppressors of yellow rust resistance on all three genomes is given by Kema *et al* (1995), working with 22 synthetic hexaploids derived from 4 *Ae. squarrosa* and 11 *T. dicoccoides* accessions. Resistance in one or both parents was frequently suppressed in the synthetic hexaploids, indicating the presence of suppressors on the AB and D genomes. Suppression appeared to be resistance gene specific, although not with all races or at all growth stages. Ma *et al* (1995) also documented resistance gene suppression by the AB and D genomes in synthetic hexaploids between *T. durum* and *T. tauschii* accessions. The resistance of certain donor parents was not expressed in the synthetic progeny, giving evidence for suppressors on both the parental genomes.

1.6.4 'Genes for Susceptibility' versus 'Suppressors' - revisited

As discussed earlier, the alternative use of the two above terms for similar phenomena is dependent upon author. This difference can probably be attributed to the means of investigation, and the means by which the identification of factors reducing disease resistance was made. The majority of the work identifying 'suppressors' of resistance has involved or been based on the introduction or combination of a number of different cultivars or accessions. Therefore, if the

expression of resistance is affected by the introduction of new genetic material, it may be assumed that the new material is actively suppressing the expression of resistance. In addition, a large proportion of the work deals with suppression that is essentially race specific, although it may be classified as resistance gene specific. It is conceptually easier to envisage race-specificity in resistance suppression as a result of suppression of individual resistance genes. Specificity of resistance genes is known and demonstrated. Therefore, specific suppression is easily modelled in this situation.

In the work describing genes for susceptibility, the authors are dealing with a single variety and apparent race non-specific suppression. The reduction in resistance can therefore be envisaged as a consequence of gene balance between resistance and susceptibility. Non-specific suppression is also more easily explained in terms of general susceptibility. Genes conferring basic, general susceptibility may be involved in some form of compatibility interaction with the pathogen, as discussed by Pink *et al* (1983). Rather than affecting the resistance genes directly, they may be providing essential products required for pathogen attack.

In reality, the two 'types' of genes may be the same. On the other hand, they may represent different classes of genes affecting disease levels in the host by different mechanisms. Without more detailed analysis of the expression and involvement of these genes in the resistance response, this distinction can not be made.

1.6.5 Null Alleles of Suppressors of Resistance

The majority of work aimed at improving disease resistance involves the introduction of resistance genes into the host. However, suppression of resistance could prevent the successful introduction of certain resistance genes. In addition, the presence of suppressors or genes for susceptibility in the host variety may reduce the level of resistance conferred by genes already

existing in the host (background resistance). An alternative means of attaining disease resistance may be via null alleles of suppressors of resistance. Dyck (1987) documented work with a gene conferring brown rust resistance, designated *LrT2*. Backcross lines of *LrT2* in the cultivar 'Thatcher' showed improved resistance to brown rust. The backcross lines also showed improved resistance to stem rust relative to the recurrent parent. It was suggested that *LrT2* may be associated with the 7D suppressor identified by Kerber & Green (1980). Substitution of *LrT2* into Canthatch nulli-7D resulted in no susceptible progeny. The lack of susceptible progeny at F2 indicated that *LrT2* was on 7D and was either closely linked with the Canthatch 7D gene responsible for suppression of stem rust resistance, or was in fact an 'null' allele of the suppressor. Evidence suggesting that *LrT2* may not be a resistance gene came from the fact that a number of accessions containing *LrT2* were susceptible to stem rust. Dyck (1987) suggests that at least some stem rust resistance would be exhibited in the susceptible accessions if *LrT2* were a resistance gene, although it is possible that *LrT2* was suppressed in these lines but not in the Thatcher background. 'Thatcher' backcross lines, having *LrT2* derived from these accessions had improved resistance to both stem rust and brown rust, indicating *LrT2* allowed the expression of 'Thatcher' resistance genes. If *LrT2* does act, possibly as a non-suppressing allele, by enhancing the expression of other resistance genes, the level of resistance exhibited by a line containing *LrT2* will depend upon the other genes present in the background.

LrT2 was designated *Lr34*. The interaction of *Lr34* with other resistance genes, leading to enhanced resistance has been demonstrated in a number of cultivars. German & Kolmer (1992) demonstrated the enhancing effect of *Lr34* in 14 pairwise combinations with seedling brown rust resistance genes in 'Thatcher' near-isogenic lines. Sawhney (1992) demonstrated that interaction of *Lr34* with *Lr27* and *Lr31* in a Chinese Spring background gave enhanced levels of field

resistance to leaf rust. *Lr34* is present in many wheat cultivars throughout the world, and is thought to play a role in durable resistance to brown rust. Interestingly, *Lr34* has also been shown to be associated with other disease resistance genes, including those for yellow rust and barley yellow dwarf virus (Singh, 1992; Singh, 1993).

1.6.6 Mutation - a means of attaining null alleles of suppressors

The potential of non-suppression as an alternative means of resistance to pathogens has been demonstrated by the *Lr34* example. The identification of suppression or genes for susceptibility in wheat provide an obvious target for resistance improvement. Inactivation of promoters of suppression would theoretically increase resistance to disease. Mutation of these genes, as proposed by a number of authors (Law *et al*, 1978; Pink *et al*, 1983; Kerber, 1991) would provide a means of improving resistance. Numerous examples of resistance as a result of mutagenisation in a number of different systems have been documented.

Inactivation of the suppressor system on 7DL of 'Canthatch' was identified by Kerber (1991) as a means of improving resistance in the parental variety. Using Ethyl MethaneSulphonate (EMS) as a mutagenising agent, a recessive null mutation was identified in two lines that permitted the expression of resistance to a number of races of stem rust to which the parental cultivar was susceptible. Analysis of F3 lines from a mutant/Canthatch cross segregated in a 1:3 ratio, indicated resistance was controlled by a recessive single gene. The resistance of the two mutants were similar to each other and to Canthatch nullisomic 7D. Furthermore, progeny from Mutant/Canthatch nulli 7D crosses were all resistant, indicating the mutation was located on 7D. There was also no segregation in a cross with Canthatch ditelosomic 7DS, indicating that mutation was located on 7DL. The mutant was crossed to the susceptible cultivar 'Columbus' (a close relative of Canthatch), and gave resistant progeny, suggesting the

mutation allowed expression of resistance genes that had previously been suppressed in Columbus. It was proposed that the mutation was at the 7DL suppressor loci or closely linked.

To eliminate the possibility that the improved resistance of the mutation was due to mutation to a resistance gene rather than non-suppression, Kerber & Aung (1995) substituted the mutant 7D chromosome into Chinese Spring. Chinese Spring and its 7D ditelocentrics are all susceptible. Therefore, in the absence of 7D, Chinese Spring expresses no resistance. If the mutation was a non-suppressor, there would be no improvement in resistance with the introduction of the 7D mutant chromosome, except for segregation of Canthatch resistance in the background. If the mutation was a resistance gene, all the progeny should be resistant. All the mutation substitution lines were susceptible, indicating that the mutation was a non-suppressor rather than a resistance gene.

The work has been paralleled by Williams *et al* (1992), again by induction of mutations in Canthatch using EMS. 15 stem rust resistant mutants were isolated that exhibited resistance to 13 races of rust. The mutations showed incomplete dominance and monogenic inheritance in crosses with Canthatch. Whilst one mutant was ditelocentric for 7DS, allelism tests of the remaining 14 mutants indicated that they were at or near the same locus on 7DL. It is therefore likely that these mutants represent further mutations in the suppressor locus first identified by Kerber & Green.

Borojevic (1974; 1978a; 1978b; 1979; 1983) carried out a comprehensive set of experiments to test the effect of mutation on inducing resistance to brown rust in a number of susceptible varieties. In several cases she noted an increase in resistance to brown rust, with race specificity being evident. The treatment of the cultivar Omar with chemical mutagens, on the other hand, yielded a number of mutants, both race-specific and nonspecific, with increased

resistance to *P. striiformis* (Line *et al*, 1974). Several of the mutants were more resistant at higher temperatures in late stages of plant growth, and as such perhaps have some similarities to the expression of adult plant resistance. Little (1971) isolated a number of induced mutants with increased resistance to *Stagonospora nodorum*, generated by either gamma rays, neutrons or ethyl methanesulfonate(EMS), of which the majority were produced with EMS. EMS was also used to produce a number of mutants resistant to either yellow rust, stem rust or mildew (Hanis, 1974) in wheat. More recently, induced wheat mutants exhibiting partial resistance to powdery mildew have been isolated (Kinane *et al*, 1996). Over 11% of the mutant lines initially identified as resistant exhibited heritable resistance to mildew, visible as reduced pustule density and conidium production. The large number of examples of artificially induced disease resistant mutants would suggest that suppressors of resistance are reasonably common in a number of cultivars. Indeed, if suppressors exist in the ancestral genomes such as *Ae. squarrosa*, as has been demonstrated by a number of authors, one might expect that suppressors are reasonably widespread throughout the wheat genomes.

The effect of mutation on disease resistance has been demonstrated in other crops as well as wheat. Mutants in barley have been characterised subsequent to treatment with physical and chemical mutagens that showed increased resistance relative to the control variety (Wiberg, 1973). The classic example of mutation induced resistance in barley is the race non-specific resistance to mildew conferred by mutations in the Mlo locus. X-ray induced mutation in the Mlo locus was first documented in barley in 1942, with naturally occurring mutants being identified in 1971. In all, more than 150 *mlo* mutant genes have been reported in a variety of backgrounds. Interestingly, the different non-complementing alleles map to one locus and *mlo* appears to be effective against all races of the pathogen (Jorgensen, 1992).

The induction of mutations clearly demonstrates an effective means of improving resistance. Whilst the introduction of resistance-improving mutations into breeding programmes may encounter problems, such as additional deleterious effects of the mutation process, mutations provide a start point for alternative sources of resistance to conventional resistance genes.

1.6.7 Hobbit 'sib' disease resistant mutants

The identification of genes for susceptibility in the variety Hobbit 'sib' by Worland & Law (1991) provided a suitable target for improvement of disease resistance. It was proposed by the authors that deletion of genes for susceptibility in Hobbit 'sib' would improve resistance, and provide markers for genes for susceptibility.

Mutation and inactivation of genes that normally reduce levels of resistance could be detected by the selection of resistant lines. RFLP identification of the deletion could then provide markers of the deletion and hence facilitate the elimination of genes promoting susceptibility from a breeding population.

Seeds of Hobbit 'sib' were irradiated with fast neutrons, and mutant lines with putatively inactivated genes for susceptibility were identified by improved levels of disease resistance relative to Hobbit 'sib'. Of the 11,000 grains initially irradiated, 20 lines were finally selected for further characterisation. It is these twenty lines that formed the basis of a great deal of the work discussed in this thesis. Full details of the selection process are detailed in Chapter 2. Results presented by the Worland & Law (1991) showed improved resistance in a subset of lines tested at M9 for yellow rust resistance (Table 1.1).

Flag leaf infection (%)				Chromosome location of mutation	
Line	Yellow rust	Brown rust	Mildew	Monosomic analysis	RFLP analysis
Hobbit 'sib'	68.3	13	7.7	---	---
Mutant I3-21	37.5***	+	+	---	5BL
I3-27	16.7***	2.5***	4.5***	---	---
I3-32	48.7***	2.0***	3.5***	5D	5D
I3-33	30.0***	+	+	---	5BL
I3-43	33.3***	2.0***	6.5*	---	---
I3-48	10.3***	1.0***	2.0***	4D	---
I3-49	9.0***	+	+	---	---
I3-54	7.7***	1.0***	0***	4B	---
I3-58	52.1	+	+	4BS	---
+ Disease reaction not available Significant difference from Hobbit 'sib'					
*P=0.05-0.01; **P=0.01-0.001; ***P= <0.001					

Table 1.1 Hobbit 'sib' disease mutant lines showing levels of infection of flag leaves to yellow rust, brown rust and mildew and the chromosomal locations of the mutation where identified by monosomic analysis or molecular RFLP analysis (after Worland & Law, 1991)

Whilst all the selected mutants were significantly more resistant than the parental control, levels of infection varied considerably between mutants, indicating improvements in resistance were probably due to mutation in different promoters of susceptibility. The mutants were tested for resistance at the seedling stage to several yellow rust races, and exhibited no race specificity (A.J.Worland, pers. comm.). Some mutants also exhibited resistance to brown rust. A single evaluation of resistance to mildew showed the selected mutants to be more resistant than the

parental control. Although the lines were selected solely on the basis of their resistance to yellow rust at the adult plant stage, some exhibited significantly improved levels of resistance to both additional diseases, although these additional results are based upon one generation test only and as such are preliminary.

Monosomic analysis was used to identify chromosome locations for the resistance of certain mutants. Detailed analysis of I3-54 was carried out. The mutant line was backcrossed on to all the Hobbit 'sib' monosomics except 3B, monosomic progeny selected and selfed, and the selfed generation evaluated for disease infection. All the chromosome families except 4B segregated plants significantly more susceptible than the mutant parent. All 40 examined plants for 4B were as resistant as the mutant, indicating the resistance, and presumably the causal mutation, was located on this chromosome. Restricted analysis of six mutants with 4B, 5BL-7BL, 4D and 5D was also carried out. The locations of resistance factors in 3 mutant lines were identified. The resistance of I3-32 was located to 5D, I3-48 to 4D and I3-58 to 4B (Table 1.1). Some RFLP analysis was conducted on selected mutants, but restricted to 24 probes located on the group 5 chromosomes. RFLP markers identified deletions on 5BL in I3-21, I3-33 and 5D in I3-32.

The use of monosomic analysis and mutant studies in Hobbit 'sib' has helped to gain an understanding of the complex genetic control of adult plant resistance in this variety. It has been shown that the level of disease on the euploid variety is probably due to a balance of genes for resistance and susceptibility. An alteration in this balance, such as the removal of susceptibility genes through either monosomy, chromosome substitution or mutation can lead to substantial improvement in the level of resistance. The removal of these genes by mutation, and the commensurate tagging of these loci would provide a handle for plant breeders to introduce this

novel form of resistance into breeding programmes. However, a great deal more work on the effects of the mutations, including their effects on other agronomic characters as well as resistance is necessary before breeders would be prepared to incorporate them into commercial breeding programs. The difficulties in utilising adult plant resistance genes in breeding programmes, as a result of the inability to recognise and handle the genes, can to a certain extent be overcome by the use of diagnostic markers attainable through the analysis of mutants. In an ideal situation, it is likely that a breeder would want to utilise only the genetic component conferring resistance from the mutant, and combine it with more favourable characteristics of other varieties. It is therefore essential to fully characterise the resistance effects of the deletions, and also a means of handling them. The mapping of such genes would also facilitate the further investigation and cloning of such loci in the long term.

1.7 Project Objectives

The objectives of this project were to locate and characterise the effects of genes for adult plant susceptibility to disease in wheat. The aims of the project can be split into two parts.

Part 1 - Description of deletion effects

The selection of mutants in the variety Hobbit 'sib' giving increased resistance to several fungal pathogens offers the possibility of locating putative genes for susceptibility, as well as possibly providing new sources of disease resistance. It was therefore of interest to assess in greater detail the effectiveness of the induced resistance of the various mutants, and to characterise the extent of the resistance to a number of diseases. A number of field trials, glasshouse experiments and controlled environment tests were therefore carried out over a 3 year period to characterise the resistance of a number of the mutants to five of the most common UK diseases, namely yellow rust, brown rust, mildew, *Stagonospora nodorum* and *Septoria tritici*.

Deletions provide a useful source of molecular markers for the null-alleles for genes for susceptibility. Several genes could have been deleted along with the gene for susceptibility. Markers for any one of these genes could therefore potentially be diagnostic for the absence of the gene for susceptibility. RFLP analysis and Representational Difference Analysis (RDA) were used to generate markers for deletions in the Hobbit 'sib' mutants. These markers were checked for co-segregation with resistance, to determine linkage. This would enable the identification of markers useful for 'tagging' the absence of the gene(s) for susceptibility. The demonstration of deletions linked to resistance in the mutant lines would also corroborate the theory of Worland & Law (1991) that mutation had inactivated genes for susceptibility in the Hobbit 'sib' parent.

Part 2 - A search for null alleles at susceptibility loci among existing varieties

The distribution of susceptibility loci in other varieties was investigated using two approaches. Analysis of Hobbit 'sib'/Bezostaya substitution lines had identified chromosomes in Bezostaya that increased the resistance of Hobbit 'sib' when substituted into the recipient variety. Some of these effects were correlated with the chromosomal locations of susceptibility genes in Hobbit 'sib' identified by monosomic analysis, and also the chromosome locations of resistance effects in certain Hobbit 'sib' mutants. The Bezostaya resistance effects could therefore be due to the Bezostaya chromosomes already carrying the null alleles of the genes for susceptibility. This was tested by crossing Hobbit 'sib' mutant lines to the respective Hobbit 'sib'/Bezostaya substitution lines to test for allelism. Allelism would indicate that both the Bezostaya chromosome and the mutant contained null alleles for the same gene for susceptibility in Hobbit 'sib'.

The presence of genes for susceptibility in several of the older varieties investigated has been demonstrated (Law *et al*, 1978; Worland & Law, 1991). Less was known of the genetic control of adult plant resistance in modern commercial varieties. It was therefore of interest to

investigate whether modern varieties contained genes for susceptibility equivalent to those identified in Hobbit 'sib', or had already been lost due to intensive breeding for resistance. The distribution of genes for susceptibility, identified using monosomic analysis of Hobbit 'sib', was investigated in seven commercial varieties. Backcross reciprocal monosomic analysis was used to compare directly five Hobbit 'sib' chromosomes shown to carry genes for susceptibility, namely 3B, 4B, 4D, 5BL-7BL and 5D, with their homologues in the commercial varieties. If the presence of genes for susceptibility could be demonstrated in current commercial varieties, these genes would provide a target for improvement of resistance in modern varieties by the removal of genes for susceptibility.

CHAPTER 2

MATERIALS & METHODS

2.1 Plant Materials

2.1.1 Variety 'Hobbit sib'

A close relative of the variety Hobbit - a semi-dwarf, high yielding, winter feed wheat bred at the Plant Breeding Institute (PBI), Cambridge. Hobbit is a cross between a number of parents, including Cappelle Desprez, Nord Desprez, CI1263, Heine 110 and Professeur Marchelle. Hobbit 'sib', also known as Dwarf A, differs from Hobbit as it contains yellow rust resistance gene *Yr1*. When first selected in 1974, Hobbit 'sib' possessed effective adult plant resistance due to *Yr14* (chromosome location not determined). It also carried seedling race specific genes *Yr1* (Macer 1966) on chromosome 2A, *Yr2* (Labrum 1980) on 7B and *Yr3a/Yr4a* (Taylor *et al*, 1981) on 5BL. Yellow rust resistance was overcome in 1975 with the evolution of yellow rust race 41E136(type 4). However, it did not become fully susceptible when all resistance genes were matched and exhibited some background resistance.

2.1.2 Hobbit 'sib' Deletion Mutants

In 1982, 11000 grains of Hobbit 'sib' were irradiated with fast neutrons at the IAEA in Vienna (Worland & Law, 1991). 2000 M1 plants showing increased resistance to yellow rust relative to Hobbit 'sib', plus a further random 2000 plants, were selected and selfed. 40 plants were selected from the M2 generation showing the highest levels of disease resistance. These consisted of two plants from each of 20 different lines. All mutant lines were checked cytologically to verify euploid chromosome number, and electrophoretic tests were carried out on grain protein extractions to ensure the mutants were identical to the parent variety in their gliadin and glutenin fractions. This was undertaken to safeguard against outcrossing - a possible source of new resistance genes.

2.1.3 Hobbit 'sib' Monosomic Series

A monosomic series (see section 2.4 for terminology) in Hobbit 'sib' was developed by backcrossing Hobbit 'sib' to the Cappelle-Desprez monosomic series using techniques described by Law and Worland (1972). Essentially, each of the 21 Cappelle-Desprez monosomics was used as the female parent and, due to differential transmission of the aneuploid chromosomes through the female and male gametes (Sears 1954), the resulting monosomic hybrids would carry a known hemizygous chromosome from the donor variety i.e. Hobbit 'sib'. By continued backcrossing with the recurrent parental variety, combined with selection for monosomic progeny, the genetic background of the developing monosomic became identical or very close to the desired donor parental variety (Fig. 2.1). At least 8 backcrosses were made.

During the development of monosomic lines it is possible for the hemizygous chromosome of the backcrossed line to change to another chromosome due to univalent shift (Law & Worland, 1996) arising from spontaneous non-disjunction of a background chromosome. Very few problems of this nature were encountered during the development of the Hobbit 'sib' monosomic series. To detect whether univalent shift had happened, the developing monosomics were used as recipient monosomics in the development of a complete series of monotelocentric plants (Fig: 2.1), in which telocentric chromosomes for each of the 21 chromosomes from the variety Chinese Spring were substituted into Hobbit 'sib'. Checking meiosis at each backcross generation ensured that, provided the substituted telocentric chromosome was unpaired, no univalent shift had occurred in the developing monosomics. The Hobbit 'sib' monosomic series was fully verified after eleven backcrosses (A.J. Worland, pers. comm).

In developing monosomics that are to be used for detailed genetic analysis, it is necessary to determine that sufficient backcrosses have been carried out to reconstitute the genetic

background of the donor variety and to lose all genes from the recipient variety. This was tested in the Hobbit 'sib' monosomics by extracting 42 chromosome progeny from selfed monosomics after 8 backcross generations and comparing to the donor Hobbit 'sib' variety. Where extracted disomics differed from Hobbit 'sib', additional backcrosses were carried out and lines were then re-checked. After 10 backcrosses, all lines appeared very similar to Hobbit 'sib'.

2.1.4 Hobbit 'sib'/Bezostaya Single Chromosome Substitution Lines

Intervarietal chromosome substitution lines carrying single chromosomes from the donor variety Bezostaya 1 (USSR bred winter wheat) substituted into a recipient Hobbit 'sib' monosomic background were used. These lines were developed at the PBI, Cambridge using backcrossing techniques described by Law and Worland (1972). Mono-telocentric plants used in the development of the Hobbit 'sib' monosomic lines to check for univalent shift were also used as the initial recipient backcross parents for the development of the Hobbit 'sib'/Bezostaya substitution lines. The telocentric chromosome of Chinese Spring was substituted for the donor Bezostaya hemizygous chromosome. The monosomic substitution line was selected and used as the male donor in the next generation, crossing back onto the developing Hobbit 'sib' monotelocentric line. After 4 or 5 backcross generations to the developing Bezostaya monotelocentric lines, backcrossing was reverted to the more homozygous background of the Hobbit 'sib' monosomics. This was repeated for up to 9 backcrosses, with selfing between generations to prevent loss of the Bezostaya chromosome due to univalent shift. Each line would be homozygous for the substituted Bezostaya chromosome in a background near to homozygous for Hobbit 'sib'. Duplicate lines were developed to provide a test for possible background variation, and also to insure against chromosome 'switch' (Law & Worland, 1996), in which rare transmission of a 20-chromosome pollen results in the substituted chromosome being replaced

by its recipient homologue. Duplication of lines also reduced the risk of loss due to human error. The development of the Hobbit 'sib' monosomics and Hobbit 'sib'/ Bezostaya substitution lines were run in tandem. The procedure is summarised in Fig 2.1.

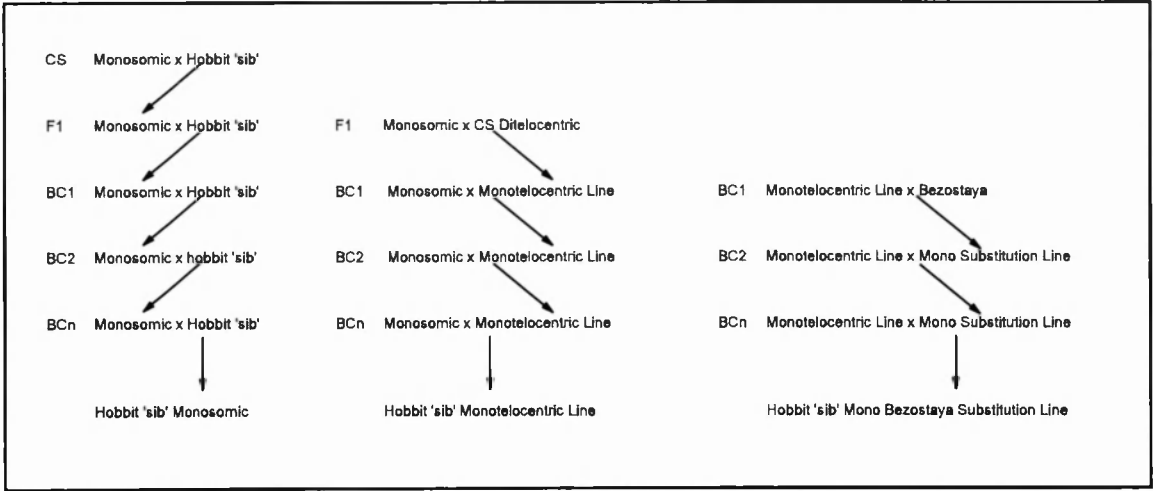


Figure 2.1: Stages in the production of monosomic lines, monosomic-telocentric lines and monosomic-substitution lines using Chinese Spring (CS) monosomics and ditelocentrics. The development of the monotelocentric lines facilitates the testing of the developing Hobbit 'sib' monosomics for univalent shift and also provides the recipient lines for the development of the Hobbit 'sib'/Bezostaya substitution lines.

2.1.5 Chinese Spring Nullisomic-Tetrasomic Stocks

Nullisomic-tetrasomic stocks are lines in which one pair of homologous chromosomes has been deleted and has been compensated for by duplicating a pair of chromosomes from the same homoeologous group, thus providing plants with a near normal phenotype. Nullisomic-tetrasomic stocks were obtained originally from Dr. E.R. Sears (Missouri, U.S.A.), (Sears, 1954), who developed them by crossing homoeologous nullisomic and tetrasomic lines of the variety Chinese Spring. This gave rise to the 42 chromosome compensating nullisomic-tetrasomic stocks.

2.1.6 Backcross Reciprocal Monosomics

Backcross reciprocal monosomic families were developed for seven varieties from the UK National List (NIAB, 1992). The euploid donor variety was first used as a male parent and crossed to the tester monosomic variety (Hobbit 'sib'). The F1 monosomics produced were then backcrossed reciprocally to the original monosomic (Fig 2.2). The resulting backcross reciprocal monosomic families differed in their hemizygous chromosomes, but their backgrounds should segregate with the same ratio of donor to recipient genes (Snape & Law, 1980). If sufficient numbers of plants were selected, the backgrounds of the two populations should be, on average, the same. Therefore, comparison of the means of the two families should predominantly reflect the differences between the hemizygous chromosomes derived from donor and recipient varieties. The F1 reciprocal monosomics were then selfed and the disomics selected in the following generation to give plants that were homozygous for one or other of the alternative homologues. Backcross reciprocal monosomics were developed for chromosomes 3B, 4B, 4D, 5BL-7BL and 5D for the varieties Genesis, Haven, Hereward, Hornet, Hunter, Rialto and Riband, using Hobbit 'sib' as the tester variety.

2.1.7 Growing of Plants for Crossing

Grains were imbibed either in 'thumb pots' containing JI No 2 compost or on moist filter paper for 24 hours at 25°C. The seeds were then incubated at 4°C for 1 - 2 days to break seed dormancy before being left to germinate at 25°C. After an 8 week vernalisation period (4 - 6°C; 9.5 hour photoperiod), the seedlings were transferred to 1 litre pots in the glasshouse (~16 hour photoperiod, supplemented with artificial light when necessary.)

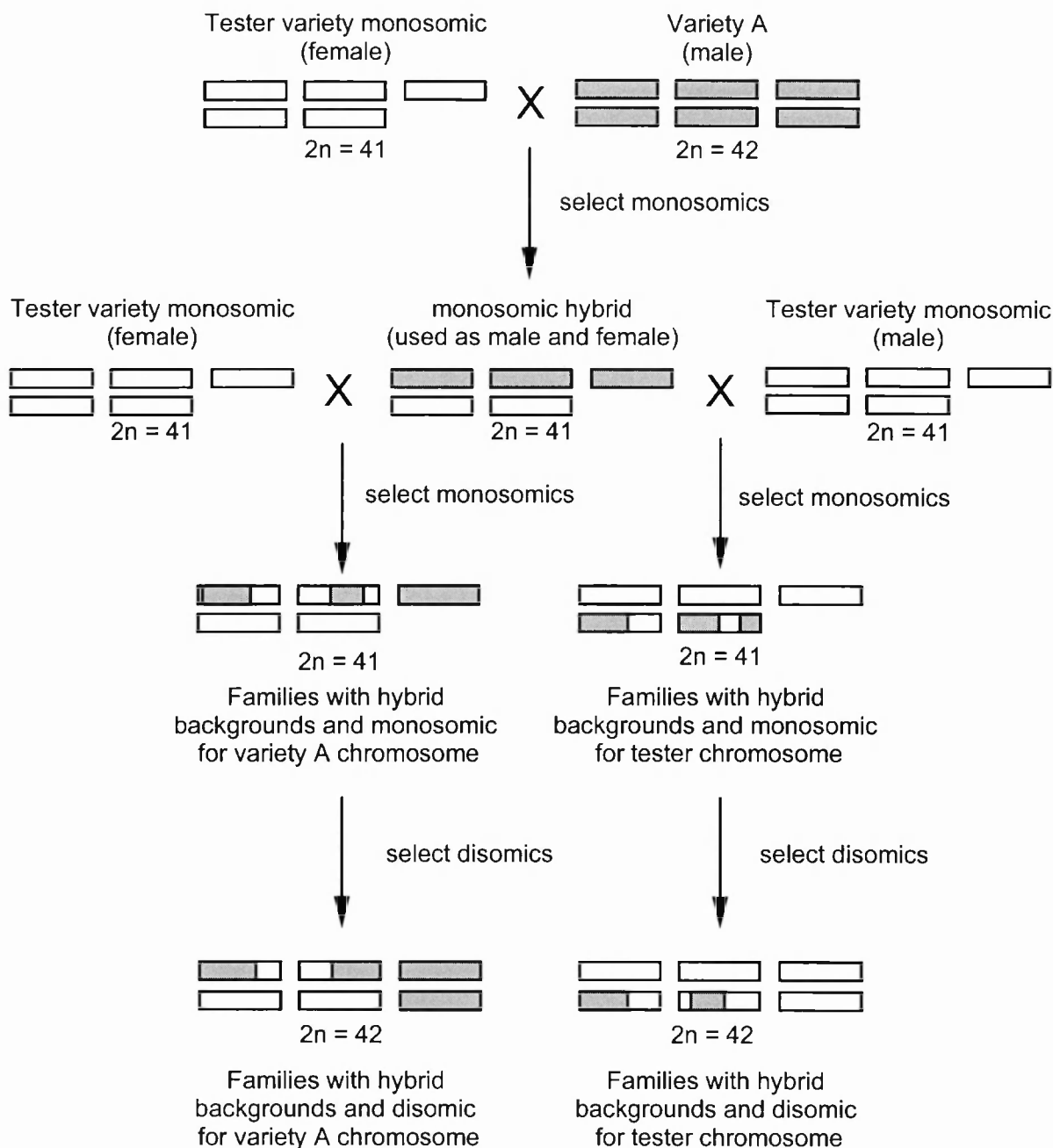


Fig. 2.2 Development of a backcross reciprocal monosomic line by backcrossing (only three pairs of homologous chromosomes shown)

2.1.8 Crossing Procedure

At flowering time (G.S 60, Zadoks *et al.*, 1974), the developing ears of female parents were emasculated using forceps to remove the immature anthers. The ears were trimmed to leave two florets per spikelet and 10 -12 spikelets per ear before covering with a cellophane bag to prevent cross-pollination. The stigmas, when expanded and receptive (usually 1-3 days later), were pollinated by dusting with ripe anthers of the male parent. The cellophane bag was replaced and left until the hybrid grains were ripe.

2.1.9 Cytogenetic Techniques for the Examination of Mitotic Chromosomes

For certain crossing procedures, monosomic plants were required. These were identified by counting the somatic chromosome numbers in root tips of germinating seeds.

Seeds were germinated on moist filter paper. When roots were about 1 cm long, two roots were removed per seed and placed in tubes containing a freshly prepared saturated solution of 1-bromo-naphthalene (BDH) in tap water. The seedlings were transferred to dishes in numerical order and stored at 4°C until those with the required chromosome number after counting could be potted on. The tubes containing the roots were left uncorked at room temperature for 4 hours 30 minutes. The 1-bromo-naphthalene solution was poured off and replaced with glacial acetic acid and the tubes corked. The roots were then stored at 4°C until they were counted. The glacial acetic acid was poured off and replaced with 1 M hydrochloric acid solution and hydrolysed at 60°C for 12 minutes uncorked. The acid was then poured off and replaced with Schiff's reagent (BDH), the tubes recorked and left to stain for approximately 30 minutes. The stained meristematic tip of the root was cut off and placed in a drop of acetic Orcein (BDH) on a slide. This was then covered with a cover-slip and tapped to break up the material. The slide and cover-slip were covered with filter paper and pressure applied to flatten the cells. Slides were examined using a

Vickers Stereomicroscope. Cells at metaphase were identified and the chromosomes were counted under oil at high magnification (x1000). Plants with 41 chromosomes were identified and selected for crossing.

2.2 Molecular Biology Techniques

Composition of underlined reagents is detailed in section 2.5.

2.2.1 Large Scale DNA Extraction

Large scale extractions were carried out using a modified method of that published by Dellaporta *et al* 1983:

1g of leaf tissue was frozen in liquid nitrogen and ground in a Mikro-Dismembrator (B. Braun Biotech) using a 20 ml PTFE vessel with 10 mm steel ball-bearings to grind the material. The material was ground for 2 minutes in the machine and transferred to a pre-chilled (liquid nitrogen) 40 ml Polycarbonate Oakridge tube (Nalgene Labware). Whole wheat ear samples were ground for 2 minutes, re-frozen in liquid nitrogen, and then ground for a further 2 minutes before transferring to the Oakridge tube.

The ground tissue was resuspended in 10 ml Extraction Buffer and 2 ml 10% Sodium Dodecyl Sulphate (SDS). This was mixed thoroughly by shaking then vortexing before being brought up to 20°C (37°C water bath for 5 minutes). 5 ml of 5M potassium acetate was added. This was vortexed then placed on ice for 20 minutes before being centrifuged at 9000rpm (10,500 x g) at 4°C for 20 minutes. The supernatant was decanted through a layer of Miracloth (Calbiochem) into a fresh Oakridge tube containing 10 ml of isopropanol. This was mixed well then placed on ice for 15 minutes prior to being centrifuged at 9000rpm (10,500 x g) at 4°C for 20 minutes. The supernatant was discarded and the pellet allowed to air -dry. The pellet was

dissolved in 0.7 ml of TNE. 20µl RNase (2mg ml⁻¹ stock) was then added and incubated at 37°C for 30 minutes, then centrifuged at 13000 r.p.m (13,400 x g) for 10 minutes. The supernatant was transferred to a fresh 1.5ml eppendorf tube containing 500 µl of isopropanol and 100 µl 3M Sodium Acetate solution. This was then vortexed and centrifuged at 13000 r.p.m (13,400 x g) for 3 minutes. The supernatant was discarded and the pellet washed in 0.5 ml 80% ethanol, then centrifuged at 13000 r.p.m (13,400 x g) for 3 minutes. The supernatant was discarded and the pellet was dried for 10 minutes under vacuum. The DNA pellet was then dissolved in 400 µl TNE or sterile distilled water (SDW).

2.2.2 Small scale DNA extraction

These were carried out using a CTAB DNA Extraction Method based on a technique described by MacDonald *et al* 1996:

50 - 100 mg of either fresh or freeze-dried leaf tissue were transferred to a 1.5ml eppendorf. 300µl CTAB buffer and a small amount of sand were added. The tissue was immediately ground using a blue disposable pestle, before incubating at 65°C for 60 minutes. 300µl chloroform were added, vortexed and centrifuged at 13000rpm (13,400 x g) for 10 minutes. The aqueous phase was added to 200 µl absolute ethanol in a new eppendorf and left at -20°C for 2 hours. After centrifugation at 13000 r.p.m (13,400 x g) for 10 minutes, the supernatant was removed and the samples allowed to air dry. The pellet was resuspended in 50 µl 1xTE+0.1% sarkosyl containing 25µl/ml RNase and incubated at 37°C for 15 minutes. 5µl 3M sodium acetate solution plus 100 µl absolute alcohol were added and vortexed before centrifugation at 13000 r.p.m (13,400 x g) for 10 minutes. The supernatant was removed and the pellet allowed to air dry. The pellet was resuspended in 20µl SDW.

2.2.3 Quantitation of Genomic DNA

5 µl of a 10-fold dilution of the resuspended DNA, in SDW and 6xloading buffer, was loaded onto a 1% agarose gel in TAE containing 0.01mg ethidium bromide. The samples were electrophoresed in TAE buffer for 20 minutes at 10V/cm. Quantitation of DNA samples was estimated by comparison with the fluorescence of λ phage DNA standards under UV light (254nm).

2.2.4 Restriction Enzyme Digestion of Genomic DNA

5 µg of genomic DNA were digested in a cocktail containing 2.5 µl 10 x restriction digest buffer, 1 µl Spermidine (0.1M stock), 2.5 µl restriction enzyme (10units/µl) and the volume made up to 25 µl with SDW. Following digestion for approximately 2 hours at 37°C, the reaction was stopped using 10 µl of 6 x Loading Buffer. Samples were loaded or stored at -20°C until required.

2.2.5 Electrophoresis of Genomic DNA for Southern Analysis

Electrophoresis of DNA was carried out in a 400ml 1% agarose gel containing 2.5 µl ethidium bromide (10mg/l) on a 20 x 20 cm platform at 30 - 40 V (1.5 - 2 V/cm) for approximately 16 hours. All gels contained one or more tracks of 0.5 µg λ HindIII DNA molecular weight marker, containing a small quantity of end-fill 32P-dCTP labelled λ HindIII DNA marker (sufficient to give clear bands after 2 days autoradiography). Gels were checked under UV light for complete digestion of samples and a polaroid photograph was taken as a permanent record.

2.2.6 Southern Blotting

Gels were immersed in denaturing solution for 45 minutes then washed for 50 minutes in neutralising solution on a shaking platform. DNA was transferred to nylon membranes (Hybond-N; Amersham International) by capillary blotting overnight in 20 x SSC, as described in Sambrook *et al*, 1989. Filters were then allowed to air-dry for at least 1 hour and the DNA

fixed by UV crosslinking for 2 minutes using a 254nm UV-transilluminator.

2.2.7 Prehybridisation

Filter prehybridisation was carried out in 35mm diameter bottles in a hybridization rotisserie incubator (HYBAID - Maxi Oven) at 65°C for a minimum of 4 hours. Filters were separated by nylon meshes (HYBAID) and were prehybridized in 15 ml/filter 1 x Denhardt's III, 1 x HSB, 0.5g/l Salmon Sperm DNA (denatured by boiling for 5 minutes immediately prior to use).

2.2.8 Probe Labelling

Probes were initially labelled by the random primer method of Feinberg and Vogelstein (1983) or later using the Rediprime DNA labelling system (Amersham International).

Random Primer Labelling:

25 - 30 ng of insert DNA was made up to 8 µl with SDW, heat-denatured (100°C for 5 minutes) and cooled on ice. After brief centrifugation to bring down contents, the following reagents were added: 3µl dNTP mix; 2µl random hexanucleotides, 2 µl Klenow Fragment of *E. Coli* DNA polymerase II, 5 µl of $\alpha^{32}\text{P}$ 3000 Ci/mmol dCTP. Incubation was at 22°C for at least 4 hours.

The probe was purified by gel filtration on Sephadex DNA Grade G-50 (Pharmacia Biotech). The G-50 resin was resuspended, poured and equilibrated in column buffer (TNE + 0.1% SDS). Total column volume was 2.7ml (10 cm high by 3mm diameter). 20 µl column dye and 200 µl column buffer was added to the probe. The probe was added to the column and 200 µl fractions were collected and monitored for activity using a hand Geiger monitor (Series 900 Type E Minimonitor; Mini-instruments Ltd.). The labelled probe usually eluted in the 2nd and 3rd fractions. Incorporation could be calculated by continuing to take fractions until the

unincorporated ^{32}P -dCTP has been eluted from the column. The activity in the incorporated peak could then be compared with that in the unincorporated peak and an incorporation value calculated. The probe was denatured by adding 1/10 volume of 3M NaOH.

Rediprime DNA Labelling

20 - 30 ng of insert DNA was made up to 45 μl with SDW, heat-denatured (100°C for 5 minutes). After brief centrifugation, the denatured DNA was added to the labelling mix. The mix was reconstituted by flicking the tube until evenly distributed before being briefly centrifuged. 5 μl of $\alpha^{32}\text{P}$ 3000 Ci/mmol dCTP was added. Incubation was at 37°C for 10 minutes. The probe was denatured by adding 1/10 volume of 3M NaOH.

2.2.9 Hybridisation of Filters

The volume of the prehybridisation solution in the rotisserie bottle containing the filter was reduced to 10 ml and the denatured probe was added. The filter was incubated at 65°C overnight in the Hybaid oven.

2.2.10 Washing of Filters

The hybridisation solution was removed from the bottle and discarded. The filters were washed for 10 minutes in about 50 ml of 6 x SSC, 0.1% SDS (pre-equilibrated at 65°C). This was to reduce background and overall radioactivity of the blots before removing the blots for the first of the stringency washes. Filters were then transferred from the bottles into 220mm x 220mm polycarbonate boxes (Stewart Plastics) containing 2 x SSC, 0.1% SDS and washed for 5 minutes at room temperature, with gentle shaking. This was followed by a 5 minute wash in 0.1xSSPE, 0.1% SDS. Where necessary, the temperature of the final wash was increased in order to reduce the counts on the filters. Filters were generally washed until the counts on the filter were between 10 and 20 cps.

2.2.11 Autoradiography

Washed filters were dried briefly on 3MM paper and were wrapped in SaranWrap (The Dow Chemical Company). They were then exposed to X-ray film (KODAK; X-OMAT, XAR5 or BioMax MR). When using the XAR5 film, two intensifier screens (Genetic Research Instrumentation Ltd) were used, arranged with film between the screens and filter externally. When using BioMax MR film, one intensifier screen (KODAK BioMax MS), arranged with the film between the screen and the filter was used. Both arrangements were placed in a side lock autoradiography cassette (Genetic Research Instrumentation Ltd.) at -70°C for 3 - 6 days. X-ray film was developed in an automatic film developer (Fuji X-ray Film Processor, RGII).

2.2.12 Generation of Cloned DNA for Radioactive labelling

Insert DNA was generated either by restriction endonuclease digestion of plasmid DNA or by PCR amplification using Forward and Reverse primers.

Plasmid DNA was prepared using the boiled lysis method as described in section 2.2.16. Purified plasmid DNA (5-10µg) was digested with 20 units of the appropriate enzyme for 1 hour to release the insert DNA. 1/6th volume of 6 x loading buffer was added and the fragments separated by electrophoresis through low melting point gels (1% agarose in TAE). Insert bands were excised and the DNA recovered with the Wizard DNA Purification kit (Promega) following manufacturer's instructions.

Direct amplification of insert DNA was carried out using PCR, with forward and reverse primers in a thermal cycler (Hybaid; Omnigene).

Forward L2 : 5' - GGA AAC AGC TAT GAC CAT GAT - 3'

$T_m = 60^{\circ}\text{C}$

Reverse L2 : 5' - GTA AAA CGA CGG CCA GTG - 3'

$T_m = 58^{\circ}\text{C}$

The reaction mix contained 2.5 ng/ml of each primer, 1.5 mM of each dNTP, 25 U/ml Taq DNA Polymerase with 1.5 mM MgCl_2 , in the Taq DNA Polymerase buffer supplied. Reactions were usually 100 μl and were overlaid with 30 μl of paraffin oil before cycling.

Cycling parameters were:-

Cycle : 94°C for 30 seconds

(30 cycles) 56°C for 1 minute

72°C for 2 minutes

Elongation : 72°C for 10 minutes

Hold : 25°C

1/6th volume of 6 x loading buffer was added and products separated on a 1% low melting point agarose gel. Bands were excised and insert purified using the Wizard DNA Purification Kit.

2.2.13 Quantitation of Insert DNA

Purified insert DNA was quantitated by comparing UV fluorescence with a set of seven known standards. For each standard, 5 μl of 2 $\mu\text{g}/\mu\text{l}$ ethidium bromide was spotted onto a petri dish. A 5 μl aliquot of the standard was mixed with the ethidium bromide. Each aliquot contained between 5 and 50 ng λ -HindIII DNA. 1 μl of insert DNA was mixed with 4 μl SDW and 5 μl of ethidium bromide and spotted on to the petri dish. The fluorescence of the spots was compared under UV light and the insert concentration determined.

2.2.14 Representational Difference Analysis

A genomic subtractive hybridisation technique based on Representational Difference Analysis as described by Lisitsyn *et al* (1993) was used to extract deletion specific clones from a number of Hobbit 'sib' deletion mutants. The subtractive hybridisation of deletion mutant (driver) and Hobbit 'sib' (tester) DNA should lead to isolation of sequences from Hobbit 'sib' that correspond to deleted sequences in the deletion mutant. The technique is summarised in Figure 4.5 (Chapter 4).

2.2.14.1 Preparation of Driver and Tester Amplicons

10 µg of both driver and tester amplicon DNA samples were digested in a final volume of 50µl with 50 units PstI restriction endonuclease, in the relevant buffer supplied by the manufacturer. 1 µg of each DNA digest was mixed with 0.5 nmol RPstI 24 and RPstI 12 in 1 x T4 DNA ligase buffer with SDW to volume.

RPstI 24 - 5'- ACT CTC CAG CCT CTC ACC GCT GCA -3'

RPstI 12 - 3'- C GGA GAG TGG CG -5'

Oligonucleotides were annealed by heating then cooling the mixture gradually from 50°C to 10°C over 1 hour. They were then ligated overnight by incubating with 6 units T4 DNA ligase at 15°C.

2.2.14.2 PCR Amplification of Driver and Tester Amplicons

80 ng of either driver or tester DNA ligation mix was added to a mixture containing 1 x React Buffer 4, 1.5 mM Mg Cl₂, 0.2 mM of each dNTP, 0.4 nM RPstI 24 and SDW to 397 µl volume. Two tubes of reaction mix were prepared for tester amplification and 10 tubes were prepared for the driver samples. The tubes were incubated at 72°C for 3 minutes in a thermal cycler (Perkin-Elmer Cetus) before 15 units Taq DNA Polymerase were added, the reactions overlaid with mineral oil and incubated for 5 minutes. The amplification consisted of 20 cycles

of 1 minute incubation at 95°C and 3 minutes at 72°C, with the last cycle followed by an extension at 72°C for 10 minutes. 5 µl of the PCR reactions were run on a 1% agarose gel. The remaining reaction mix was purified using a Wizard DNA Purification Kit (Promega) to remove unutilised primers and quantitated by measuring UV fluorescence compared to a series of standards.

2.2.14.3 Hybridisation of Tester and Driver Amplicons

1 µg of the tester amplicon was digested with PstI (10U/µg) in 100 µl volume to remove the RPstI 24 and RPstI 12 primers. The products were purified using the Wizard kit, and then requantitated. 500 ng of tester amplicon was ligated to new adaptors as described in section 2.2.14.1.

JPstI 24 - 5'- GAC GTC GAC TAT CCA TGA ACT GCA -3'

JPstI 12- 3'- G ATA GGT ACT TG - 5'

The ligated tester amplicon was added to 40 µg of driver amplicon, and precipitated with 1/10 volume 10M ammonium acetate and 2 volumes 100% Ethanol. This was then vortexed, placed at -20°C for 20 minutes and then centrifuged at 13000 r.p.m (13,400 x g) for 20 minutes. The supernatant was discarded and the pellet washed in 0.5 ml 80% EtOH, then centrifuged at 13000 r.p.m (13,400 x g) for 10 minutes. The supernatant was discarded and the pellet was dried for 10 minutes under vacuum. The DNA pellet was then dissolved in 4 µl 3x EE (Straus & Ausebel, 1990) and overlaid with mineral oil prior to boiling for 5 minutes. 1 µl of 5M NaCl was added and the mixture incubated overnight at 65°C.

2.2.14.4 PCR Amplification of Hybridisation Reaction

The hybridisation reaction mix was diluted to 20 µl using SDW. 2 µl of this reaction were added to a mix containing 0.2 mM of each dNTP, 1.5 mM MgCl₂, 15 U Taq DNA Polymerase made to 394 µl with SDW, overlaid with mineral oil. Following incubation at 72°C for 5 minutes

0.4 nM JPstI 24 was added and the reaction mix amplified for 10 cycles (1 minute, 95°C; 3 minutes, 70°C) followed by an extension period at 72°C for 10 minutes. This was followed by a 30 minutes incubation at 37°C with 20 U of mung bean nuclease to digest single stranded DNA, in a volume of 40 µl, diluted (1:5) in 50 mM Tris-HCl (pH 8.9). The enzyme was inactivated by incubating at 95°C for 5 minutes.

40 µl of the this reaction was added to a second amplification mix containing 0.2 mM of each dNTP, 1.5 mM MgCl₂, 15 U Taq DNA Polymerase, 0.4 nM JPstI 24 and made to 400 µl with SDW. This was amplified for 20 cycles (1 minute, 95°C; 3 minutes, 70°C) followed by an extension period at 72°C for 10 minutes.

10 µl of the PCR products was run on a 1% agarose gel. The remaining product was purified using a Wizard kit and quantitated as in section 2.2.14.2.

2.2.14.5 Second Round Subtractive Hybridisation

The steps described in sections 2.2.14.3 and 2.2.14.4 were repeated, except that only 400 ng of tester amplicon were used (the product of section 2.2.14.4) and were ligated to adaptors NPstI 24 and NPstI 12.

NPstI 24- 5'- CAA CTG TGC TAT CCG AGG GCT GCA -3'

NPstI 12- 3'- G ATA GGC TCC CG -5'

The products of the second round were run on a 3% Metaphor gel (FMC Products) with 0.01 mg EthBr in TBE for 4 -6 hours at 100 V (10V/cm). The products were visualised under ultra-violet light (240nm) and photographs were taken.

2.2.14.6 Cloning of Amplified Products

50 µl of the final amplification were digested at 37°C for 1 hour with 10 U PstI in the appropriate buffer made to 100µl volume with SDW. The product was purified using the Wizard

kit, and quantitated.

10 µg of pUC 19 vector DNA was digested with an excess of PstI for 1 hour at 37°C and the product DNA was gel purified. 100ng of vector DNA was ligated to approximately 30 ng of amplified product using 1µl (1U) of T4 DNA Ligase in 2 µl of the buffer supplied (made up to 10 µl with SDW) at 15°C for at least 16 hours.

1 µl of ligation mix was transformed into competent *E. coli* cells (Max Efficiency DH5a; GIBCO-BRL) by heat shock according to the manufacturers recommendations. Selection for cells containing transformed plasmids containing inserts was carried out on Luria Broth plates containing ampicillin (1 µg/µl) and X-gal (0.04 µg/µl), followed by incubation overnight at 37°C.

2.2.15 Boiled Miniprep Plasmid Purification

The selected colonies were amplified overnight in 2 ml Luria Broth cultures containing ampicillin at 37°C on an orbital shaker. The culture was then decanted into an Eppendorf tube and centrifuged at 13000 rpm (13,400 x g) for 1 minute. All but 10 µl of the supernatant was discarded and the pellet was resuspended in the remaining supernatant. To this was added 375µl M-STET plus 7µl lysozyme (40 µg/ml) and vortexed for 3 seconds. The solution was boiled for 1 minute followed by centrifugation at 13000 rpm (13,400 x g) for 15 minutes at 4°C. The pellet was removed using a sterile toothpick. 1 volume isopropanol and 1/10 volume 3M NaAc was added to the supernatant and mixed thoroughly prior to incubation for 10 minutes at -20°C and spun at 13000 rpm (13,400 x g) for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed in 80% ethanol, dried under vacuum and resuspended in 100 µl TNE, containing 2 µl RNase. After a 15 minute incubation at 37°C, 2 volumes ethanol and 1/10 volume 3M Sodium acetate were added, mixed thoroughly and left at -20°C for 10 minutes before centrifugation at 13000 rpm (13,400 x g) for 10 minutes. The pellet was washed and dried as

above and then resuspended in 50 µl SDW.

The plasmid was digested with suitable enzymes using the appropriate buffer at 37°C for one hour. Loading buffer was added to the digested DNA and run on a 1% agarose gel at 100V (10V/cm) for 45 minutes. The gel was viewed over UV light and the size of bands determined. The size of DNA fragments was estimated by comparison against standard molecular weight markers.

2.3 Disease Inoculation

All disease isolates were sourced from the Cereal Pathology department, PBI Cambridge. All isolates were stored in liquid nitrogen prior to use, except the mildew isolates which were maintained on living plants. Information detailing the virulence of the isolates was supplied by A.J. Taylor, PBI Cambridge (pers. comm).

2.3.1 Yellow Rust Inoculation

Depending upon growth stage or environmental conditions, one of two methods of inoculation was used.

Talcum Powder Based Inoculation:

This method was used to inoculate seedlings at the 2 leaf stage (Growth Stage (GS) 12, Zadoks *et al*,1974) to transfer inoculum to the field trials. It was also used for inoculation of adult plants (GS 37 - 41) for glasshouse tests.

Plants were inoculated with a mixture of spores in talc (Sigma), in a ratio of 1:15 spores:talc. 0.1 g of spores were used per 1000 seedlings or per 60 adult plants. Plants were first wetted with a fine spray of distilled water. The spore/talc mix was blown onto to the plants using a hand held powder blower. The plants were then incubated at 10°C for 24 hours (dark) at 100%

relative humidity before being transferred to greenhouse compartments (approximately 15 hours light per day; 18 - 20°C).

Odourless Kerosene Based Inoculation:

This method was used to inoculate field trial material nearing adult plant status (GS 37 - 41). Plants were inoculated directly with 0.1g spores suspended in 20 ml odourless kerosene (Woburn Chemicals Ltd). The suspension was dispensed using a Microulva battery powered sprayer (Micron Sprayers Ltd) at a rate of 1ml per plot (1m x 0.6m).

2.3.2 Brown Rust Inoculation

Brown rust inoculation was carried using talcum powder based inoculation as described previously, except the incubation conditions were changed to 15°C for 48 hours (dark) at 100% relative humidity.

2.3.3 Mildew Inoculation

Seedling and adult plant material was inoculated using a direct spore dispersal method.

Plant material to be tested was infected by shaking spores from infected plants directly onto the plants. This was repeated several times over a period of a few days from the first inoculation to ensure successful inoculation.

2.3.4 Stagonospora nodorum Inoculation

Inoculation of field material was carried out at growth stage 37 - 41 (Zadoks *et al*, 1974) and at growth stage 55 -59. Inoculum was carried in suspension at a concentration of 1×10^6 spores per ml distilled water. The suspension was applied at a rate of 22ml per m². The high humidity required for infection was maintained using fine mist irrigation (2 minutes mist, every 30 minutes from 8.00 am to 6.00 pm).

2.3.5 *Septoria tritici* Inoculation

Inoculation of field material was carried out as in section 2.3.4, except that initial inoculation occurred at growth stage 32 - 33 with a second inoculation at growth stage 37 - 41.

2.3.6 Rust Field Trials

Rows of genotypes to be tested were sown adjacent to a row of a susceptible spreader (usually var. 'Vuka' for yellow rust), using a 'Spider' self propelled drill. Each 1.2m x 0.6m plot consisted of four test rows, with a spreader row between each pair of test rows (Fig. 2.3). Approximately 10 - 20 seeds were planted per 0.6m row, with approximately 0.2m spacing between each row. Spreader rows were usually inoculated by transplanting infected seedlings produced in the greenhouse (see sections 2.3.1 and 2.3.2) into the row. This was done in the spring (March/April) at spacings of 2 seedlings per 0.6m row.

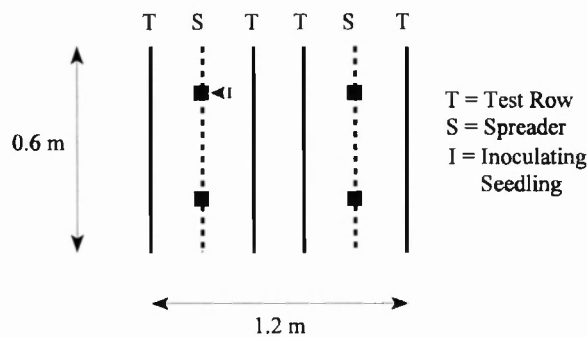


Figure 2.3: Plot layout for rust field trials

2.3.7 *Septoria tritici* and *Stagonospora nodorum* Field Trials

Plots were sown to the same dimensions as described in section 2.6.1, but with the absence of spreader rows. Four test rows were planted adjacent to each other in the central four rows of the plot, with a guard variety (var. 'Brigadier') planted on the edge rows (Fig. 2.4).

Inoculation was as described in sections 2.3.4 and 2.3.5.

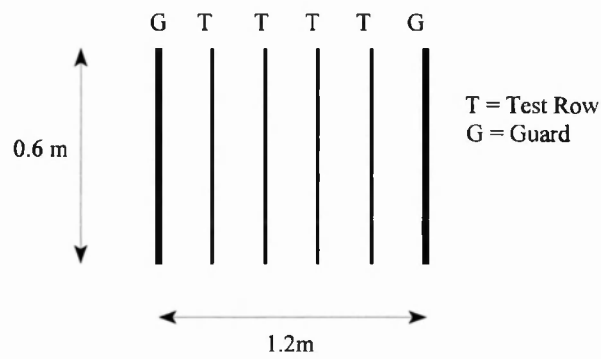


Fig 2.4: Plot layout for *S. tritici* and *S. nodorum* field trials

2.3.8 Growth Chamber Tests

Adult plant disease tests were carried out in controlled environment chambers. Seedlings were vernalised (4 - 6°C; 9.5 hour photoperiod) for 8 weeks before being transferred to the growth room and grown in 1 litre pots containing John Innes No.2 compost.

The plants were grown with a 16 hour photoperiod and light intensity of 170µmol m⁻² sec⁻¹. Day and night temperatures were 20°C and 12°C respectively. Inoculation was as described in section 2.3.3.

2.3.9 Glasshouse Tests

Adult plant disease tests were also carried out under glasshouse conditions. The plants were grown with a 16 hour photoperiod, with 25°C day, 10°C night temperatures as far as was practically possible.

2.4 Nomenclature relating to aneuploid stocks

In the variety Chinese Spring, a complete series of plants, in which the dosage of

individual chromosomes varies from 0 to 4, were developed by Sears (1954). In addition, certain stocks lack particular chromosome arms. The terms relating to these stocks are described below:

Euploid -	Describes lines with the full chromosome complement (n=42).
Monosomic -	Describes lines with a single chromosome missing (n=41).
Nullisomic -	Describes lines with a chromosome pair missing (n=40).
Tetrasomic-	Describes lines with an additional chromosome pair (n=44).
Monotelocentric-	Describes lines with a single chromosome arm present as a monosomic.
Ditelocentric -	Describes lines with a pair of chromosome arms missing.
Nullisomic/tetrasomic -	Describes lines lacking one chromosome pair, but containing a duplicate pair of another chromosome from the same group such that the chromosome number is the same as the euploid.

2.5 Stock Solutions

Stock solutions which were autoclaved are indicated by (A). The autoclave cycle was 15 lb/in² at 121°C for 30 minutes. The entire cycle took approximately 2 hours. All stocks were stored and pH balanced at room temperature, unless otherwise stated.

1. **Extraction Buffer** - 0.1 M Tris, 0.05 M EDTA, 0.5 M NaCl, 1% PVP 40, pH 8.0 (A); add β -mercaptoethanol to 10 mM prior to use (Dellaporta *et al*, 1983).
2. **TNE** - 20 mM Tris, 20 mM NaCl, 1mM EDTA; pH 8.0. (a)

3. **CTAB buffer** - 20 mM Hexadecyltin-methylammonium bromide, 135 mM Sorbitol, 35mM Sarkosyl, 0.8 M Sodium chloride, 20 mM EDTA, 0.25 mM PVPP.
4. **6 x Loading Buffer** - 15% ficoll type 400, 0.025% bromophenol blue, 0.1 M EDTA. Store at -20°C.
5. **TAE** - 40 mM Tris base, 20 mM acetic acid, 2 mM EDTA; pH 8.0.
6. **32P-dCTP labelled l-HindIII DNA Marker** -
1 µg HindIII cut l-DNA (1ml), <1 µλ (<10 mCi) 3000 Ci/mmol 32p - dCTP, 2 µl Reaction Buffer 1 (GIBCO -BRL), 1 µl (10 U) Klenow DNA Polymerase, SDW to 20 µl total volume; Incubate at 37°C for 30 min. Stop reaction with 10 mM EDTA and SDW to 200 µl. Store at -20°C.
7. **Denaturing Solution** - 0.5 M sodium hydroxide, 0.15 M sodium chloride.
8. **Neutralising Solution** - 0.5 M Tris, 3 M sodium chloride.
9. **20 x SSC** - 3M sodium chloride, 0.3M sodium citrate.
10. **10 x Denhardt's III Stock** - 2% gelatine, 2% Ficoll - 400, 2% PVP - 360, 5% sodium

pyrophosphate, 6% SDS; Leave stirring O/N to dissolve.

Store at -20°C.

11. **5 x HSB Stock** -

3M NaCl, 0.1 M PIPES. 20 mM EDTA; pH 6.8. (A)

12. **Salmon Sperm DNA** -

5 g in 1 litre ; dissolve with stirring. Store -20°C.

13. **Random Primer Labelling dNTP mix** -

0.3 mM dATP, dGTP and dTTP made from 100 mM stocks (Pharmacia) with SDW and stored at -20°C.

14. **20 x SSPE** -

3M sodium chloride, 0.2 M sodium dihydrogen orthophosphate, 0.02 M EDTA.

15. **M-STET** -

50 mM EDTA, 50 mM Tris, 5% sucrose/50 mM tris Cl (pH8.0)

16. **1 x TE + 0.1% Sarkosyl** -

50 mM Tris, 10 mM EDTA, 0.1% Sarkosyl; pH 8

17. **Luria Broth** -

20 g per litre of LB Broth Base (GIBCO-BRL); (A)
Store at 4°C

18. **Ampillin** - 100 mg/ml aqueous solution; store at 20°C.
19. **X-gal** - 2% w/v 5-Bromo- 4-Chloro- 3-Indoyl b-D Glucuroniside in n,n-Dimethyl fromamide (DMF); Store at -20°C in dark.
20. **Column dye** - 0.1 mg Blue Dextran 3000 (Sigma), 0.1 mg Orange G in 20 ml TNE.
21. **TBE** - 89mM Tris-borate, 2 mM EDTA; pH 8.
22. **3 x EE** - 30 mM EPPS, 3 mM EDTA; pH 8.

CHAPTER 3

THE DISEASE RESISTANCE OF HOBBIT 'SIB' MUTANT LINES

3.1 Introduction

In previous work by Worland & Law (1991; unpublished), irradiation treatment resulted in an increased level of resistance to yellow rust, relative to Hobbit 'sib', in a number of mutant lines. At the M1 generation, 2000 plants that appeared to show less than average levels of rust infection, plus a further 2000 randomly selected plants were selfed and the progeny tested at M2. 40 M2 plants showing the highest level of disease resistance were selected. 32 of these lines were selected as resistant relative to Hobbit 'sib' at M1 whilst 8 lines were derived from randomly selected plants. In the M3 and M4 generations, the lines were further screened in fully randomised experiments for resistance to yellow rust (*Puccinia striiformis*). At M3, all families were checked to determine chromosome number and glutenin and gliadin banding patterns relative to the Hobbit 'sib' control. Following such checks, 8 lines were discarded as they appeared to be off types or outcrosses. A further 12 lines were rejected due to aneuploidy or relatively high disease levels. Of the remaining 20 lines at M4, nine showed segregation in infection levels, although the more susceptible segregants were usually more resistant than the control. 13 lines were further tested at M5 and M6, with all lines showing significantly improved levels of resistance at M6, despite relatively high levels of infection in 4 lines at M5. In later generations, from M7 to M10, the 5 most resistant lines were screened for resistance to yellow rust, brown rust (*Puccinia recondita*) and mildew (*Erysiphe graminis*). The improved levels of yellow rust resistance previously noted were confirmed. Evaluation of brown rust resistance at M8 and M9 showed an improved, usually significant, level of resistance to this disease. A single evaluation of mildew resistance also showed the mutants to be more resistant than the parental control.

Significant resistance effects in a number of mutants were thus clearly demonstrated

(Table 1.1). Whilst full characterisation of yellow rust resistance was carried out on 20 mutant lines to M4, subsequent generations were only partially characterised, with tests for brown rust and mildew being undertaken on only a small number of mutants, and in only one case for mildew. It would therefore be interesting to understand the resistance response of the whole series to a number of diseases, including not only obligate pathogens such as yellow rust, but also facultative pathogens. Full characterisation of the mutants is also interesting from the perspective of establishing whether the deletion mutants have a common resistance response, or whether the resistance phenotypes vary, with different deletions being responsible.

The work presented in this chapter details a series of field trials carried out over a three year period to characterise and compare the levels of resistance exhibited by 21 selected mutant lines with the Hobbit 'sib' parent. The mutants were tested in the field with 4 diseases: yellow rust, brown rust, *Stagonospora nodorum* and *Septoria tritici*. A small number of mutants, including the 5 lines previously characterised at M7 to M10, were tested for mildew resistance under controlled conditions in a growth cabinet.

3.2 Pathogens

Various field and growth cabinet tests were carried out with the pathogens detailed below.

3.2.1 Yellow Rust

Yellow rust (*Puccinia striiformis*) forms distinctive stripes of yellow sporulating uredial pustules. Adult plants were scored by estimating the percentage flag leaf cover of these stripes at growth stage 54 (Zadoks *et al*, 1974). Further scores at weekly intervals were made up to about growth stage 65. At low infection levels, single pustules are visible, developing into stripes at intermediate levels, with coalesced areas of infection at high levels (Fig: 3.1).

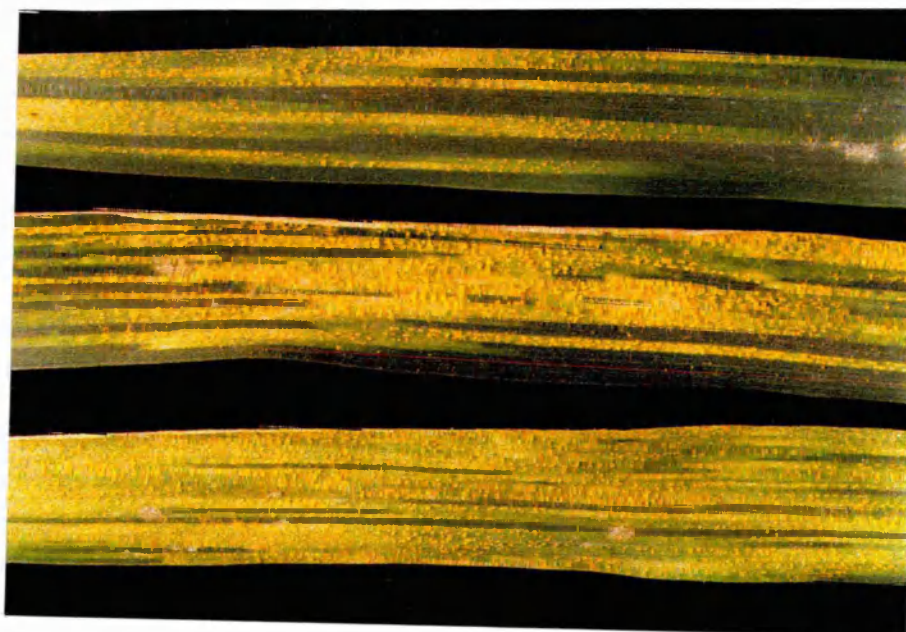


Figure 3.1: Yellow Rust Pustules on Wheat Flag Leaves

All mutant lines were tested with a single race isolate, 41 E 136(4), with virulence to the major resistance of the parental variety, 'Hobbit sib' determined by the genes Yr1, Yr2, Yr3a/Yr4a and Yr14.

3.2.2 Brown Rust

The symptoms of brown rust (*Puccinia recondita*) are identifiable as small, oval-shaped, red to brown pustules of uredia scattered across the leaf (Fig: 3.2). Infection levels were measured by estimating percentage flag leaf area covered at similar growth stages as the yellow rust, although due to the later development of the disease, the first score occurred nearer growth stage 60.

A mixture of four races was used containing the following isolates: 90-12; 92-15; 93-37; 94-21. These were all isolated from UK winter wheat populations and are known to attack a wide range of winter wheats.



Figure 3.2: Pustules of Brown Rust on Flag Leaves

3.2.3 Mildew

Mildew (*Erysiphe graminis*) infection is distinguished by fluffy, white superficial mycelial colonies (Fig: 3.3).

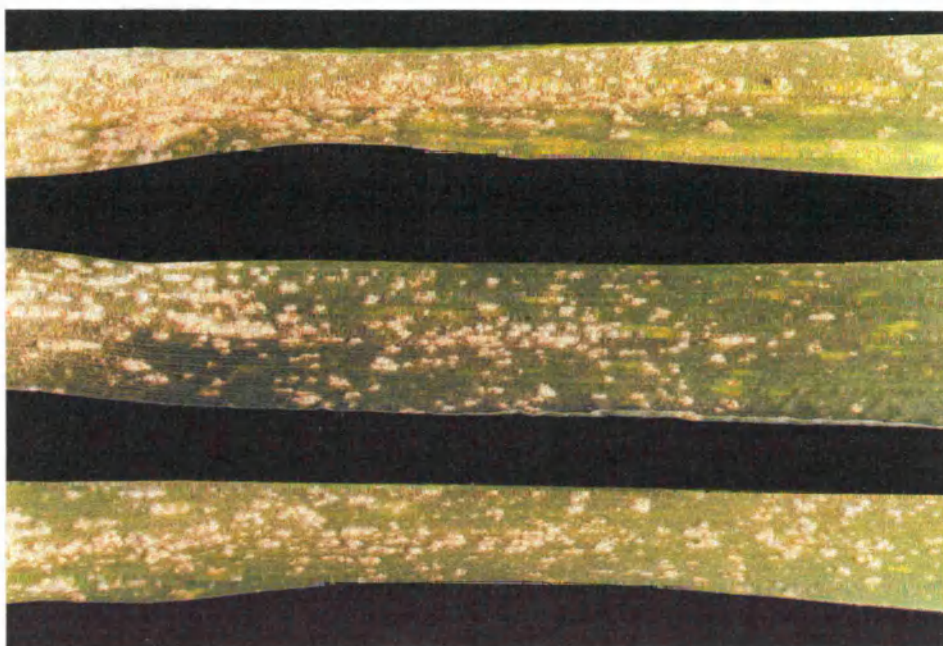


Figure 3.3: Mildew Conidia on Flag Leaves of Wheat

A vigorous isolate with wide virulence was used in growth cabinet tests of selected mutants. Adult plant infection was estimated by measuring percentage flag leaf cover at approximately growth stages 60 and 65. The isolate has specific virulence to the following major resistance genes: Pm1, Pm2, Pm3a, Pm4b, Pm5, Pm8, Pm11m Mli, MLk, Pm6, Pmx, Mld.

3.2.4 Septoria tritici

Symptoms first appear as small, irregular reddish brown leaf spots that tend to develop longitudinally, due to restriction by the veins of the leaf. The centres of the lesions become ash coloured. The lesions expand and merge, with distinctive dark specks (pycnidia) appearing. Infection levels were determined by estimating percentage flag leaf cover and whole plant infection at approximately growth stage 59 - 60, with further scores at weekly intervals until the disease reached completion or finished spreading.

A five isolate mixture supplied by A.J. Taylor, PBI Cambridge was applied, comprising: S362/90; S368/91; S381/93; S383/93; 452/95. These were all isolated from UK winter wheats and are known to attack a wide range of varieties.

3.2.5 Stagonospora nodorum

The blotches caused by *Stagonospora nodorum* are less restricted than *Septoria tritici*. *S. nodorum* tends to form yellowish to tan, oval lesions. As the lesions enlarge and merge, the necrotic regions become light grey in colour. Light brown pycnidia develop although are less conspicuous than those of *S. tritici*. As the disease develops, it becomes increasingly similar in appearance to *S. tritici* so great care must be taken when scoring leaf area infection. The disease was scored at similar stages to *S. tritici*.

A four isolate mixture supplied by A.J. Taylor was applied, comprising: S52/73/1; S372/92; S454/95; S455/95. All isolates were obtained from UK winter wheat varieties.

3.3 The Hobbit 'sib' mutant lines

The Hobbit 'sib' mutant lines were selected, maintained and evaluated for a differing number of generations from 3 (M3) to ten (M10) generations (Worland & Law, 1991; Worland *et al*, unpublished). The mutant lines used in this study will have therefore been subject to different levels of selection. The number of generations of evaluation carried out for each mutant (Worland *et al*, unpublished) are detailed in Table 3.1.

Final generation of mutant evaluation	Hobbit 'sib' mutant(s)
M3	I3-23
M4	I3-21, I3-26, I3-30, I3-33, I3-35, I3-36, I3-42,
M6	I3-20, I3-31, I3-34, I3-37, I3-55, I3-58, I3-59
M7	I3-49
M10	I3-27, I3-32, I3-43, I3-48, I3-54

Table 3.1: The extent of evaluation carried out for the Hobbit 'sib' mutants, showing the final generation of evaluation and the mutants evaluated to that generation.

3.4 Disease Tests Layout

All field trial plots were given standard farm treatments of herbicides but no fungicides. Nitrogen was applied at 180 kg/Ha.

3.4.1 Yellow Rust Trials

Field experiments were grown at PBI Cambridge in 1993/94, 1994/95 & 1995/96. 21 mutant lines were sown, together with 3 Hobbit 'sib' controls, drilled as rows in 2 replicate blocks in 1993 and 3 replicate blocks in 1994 and 1995. Lines were randomised within the block, with 4 rows sown per plot (Chapter 2.3; Fig. 2.5).

An additional field trial was also sown at Morley Research Station, Norfolk in October

1995. 17 lines plus 1 Hobbit 'sib' control were sown as single, randomised rows within two replicate blocks. A 1m strip of the variety 'Vuka' was sown alongside each strip of experimental material to act as a yellow rust spreader. The susceptible spreader rows of all experiments were artificially infected with yellow rust by transplanting infected and sporulating seedlings into spreader rows in early spring.

3.4.2 Brown Rust Trials

Field experiments were sown at PBI in October 1994 and 1995. 21 mutant lines plus 3 Hobbit 'sib' controls were sown in 3 replicate blocks, randomised within each block such that each block contained 4 rows of 6 plots each. Spreader rows were inoculated using transplanted infected seedlings in early spring.

3.4.3 Septoria tritici and Stagonospora nodorum Trials

Field trials were sown at PBI in October 1993 -1995 in the same block layout as brown rust, with plot layouts as detailed in Chapter 2.3.7. The trials were sown as 4 replicate blocks in 1994 and 1995.

3.4.4 Mildew Tests

Tests were carried out on 7 selected mutant lines, namely I3-21, I3-27, I3-32, I3-43, I3-48, I3-54 and I3-58, plus a Hobbit 'sib' control. These mutant lines were selected as they had exhibited good levels resistance to yellow rust and were the lines showing most stability of infection type within experimental rows(A.J. Worland - Pers. Comm.). Ten plants of each line were grown under controlled conditions in growth cabinets as described in section 2.3.8.

3.5 Results and Discussion

Results were analysed using the statistical package GENSTAT 5. Analyses of variance were performed on the data collected from separate field trials over the 3 year period. The characters measured in each trial are detailed in each disease sub-section.

3.5.1 Yellow Rust

There were significant differences between most of the different mutant lines and the Hobbit 'sib' parental control, trialled at PBI Cambridge, as illustrated in Fig. 3.4. The majority of the mutants exhibited a significantly increased level of resistance. A total of twelve lines showed significant resistance throughout all three years of trialling. These were I3-21, I3-26, I3-27, I3-30, I3-32, I3-43, I3-48, I3-49, I3-54, I3-55, I3-58 and I3-59.

Lines I3-30, I3-48 and I3-49 have shown extremely low levels of infection relative to the control throughout the three years, with the flag leaf infection rarely being more than 5%. In glasshouse trials, Hobbit 'sib' reached infection levels as high as 60%, whereas I3-48 had flag leaf infection levels of around 2%, indicating the high levels of resistance exhibited by this mutant.

Three lines exhibited levels of infection below 10% flag leaf coverage, namely I3-27, I3-43 and I3-54. The remaining consistently resistant lines were still considerably more resistant than Hobbit 'sib', with flag leaf infection levels of no more than 50% of the Hobbit 'sib' control level.

Lines I3-34 to I3-37 were omitted from the 1995 trial as there was a lack of seed at the time of trial preparation. However, in other years the four lines exhibited reasonable levels of resistance. With further disease testing for another year, it may be possible to demonstrate consistent resistance in these four lines as well.

Due to the highly necrotic nature of the mutant line I3-33, an accurate estimate of disease

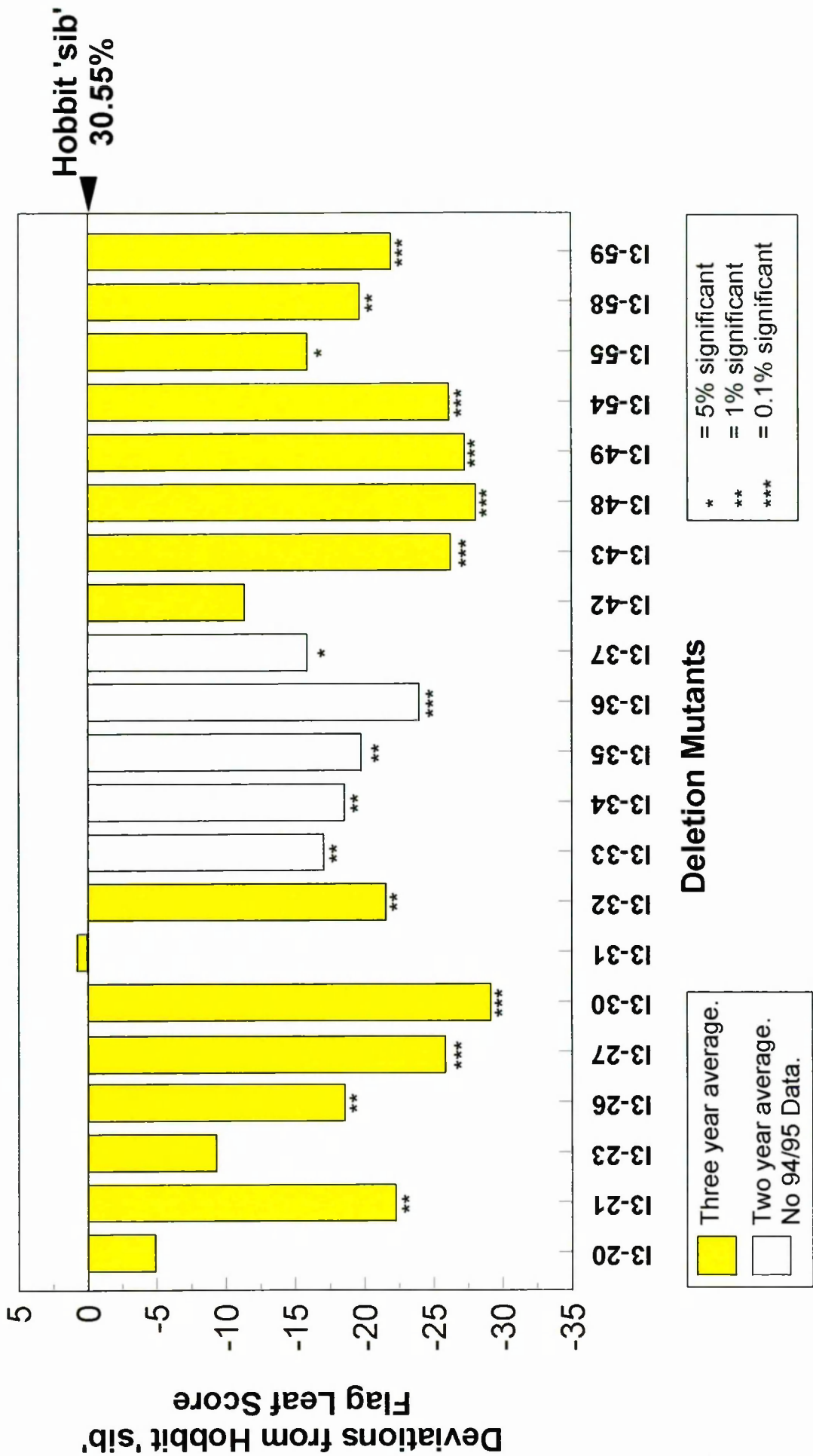


Fig 3.4: Average %flag leaf yellow rust infection on 21 Hobbit 'sib' mutants, relative to Hobbit 'sib', showing a significant reduction in infection for 17 mutants. Each score represents a 3 year average infection at PBI Cambridge, except for those mutants shown in white, where the score represents a 2 year average.

infection could not be determined in the 94/95 trial. The line was therefore omitted from the analysis for that year. The line I3-33 was only investigated to M4 in the original investigation by Worland *et al* (unpublished), and demonstrated varying levels of disease response within the line. This was subsequently shown to be due to segregation of aneuploids in the line. The most resistant plants in the initial study showed a significant improvement in resistance relative to Hobbit 'sib', much in line with the 93/94 and 95/96 results of this study.

I3-23 showed increased levels of resistance in the 93/94 and 94/95 trials, but was more susceptible than the control in 95/96. This may be due to contaminated seed stock or might be due to loss of the deletion as several mutant lines were not selected to complete stability (A.J. Worland, Pers. Comm.). The variation in resistance response of this line confirms the correct classification of this line as an off-type in previous studies.

Mutant line I3-31 was highly susceptible in 1994. However, this result was removed from the analysis as examination of the line in the field at the time of scoring indicated that it was an aberrant seed stock that appeared as if the 5BS-7BS chromosome had been lost (nullisomic 5BS-7BS). The loss of this chromosome would result in the loss of the strong adult plant resistance genes it carries (Johnson & Law, 1973). Analysis of the remaining two years data, derived from an alternative seed stock, showed no significant reduction in disease level relative to Hobbit 'sib'.

Although the 21 mutant lines were originally selected from the 40 most yellow rust resistant lines at M2, it will be observed that only 12 mutants continue to show a consistent positive resistance response. Other than those exceptions discussed above, two non-consistent lines remain. I3-20 was significantly more resistant only in the 93/94 trial, whilst I3-42 was resistant in the 94/95 trial only. Two possible explanations of the 'loss of resistance' can be considered.

(i) New race emergence

Worland & Law (1991) showed that the mutants exhibited no seedling race specific differences. However, it is possible that a more recently evolved race might have some race specific effect. The relatively stable level of infection on the Hobbit 'sib' control over the three years of trials would suggest that there has been no new race emergence affecting resistance levels in these trials. However, the resistance of certain mutant lines may still be race-specific

(ii) Segregation of mutant lines

It is known that the loss of resistance in some lines in the initial study by Worland & Law was due to continued segregation of the lines after initial selection. One could consider that the mutants were selected at the equivalent of an F₂ generation, and thus were heterozygous. It is well known that even major genes do not necessarily become fixed for a number of generations, with polygenic characters taking even longer, depending upon the number of genes involved (Law & Worland, 1996). Therefore, the resistance effect selected in the original I3-20 and I3-42 lines may well have been lost in subsequent generations. Amongst the lines tested, it is known that some were subjected to more rigorous and extensive selection after the original M₂ selection. It was known that many of the mutant lines were definitely unstable at the M₃ and M₄ generations (A.J.Worland - pers. comm.). Hence the selection of a subset of 5 mutants (I3-27, I3-32, I3-43, I3-48 and I3-54) for further investigation in the initial study. All these lines have remained resistant in the current investigation.

Additional trial at Morley, Norfolk

A single yellow rust trial of 17 mutant lines was carried out at Morley Research Centre, Norfolk in 1994/1995. The lines I3-34 to I3-37 were not included. As illustrated in Fig. 3.5, 16 of the 17 lines tested were significantly more resistant to yellow rust. As with the PBI Cambridge

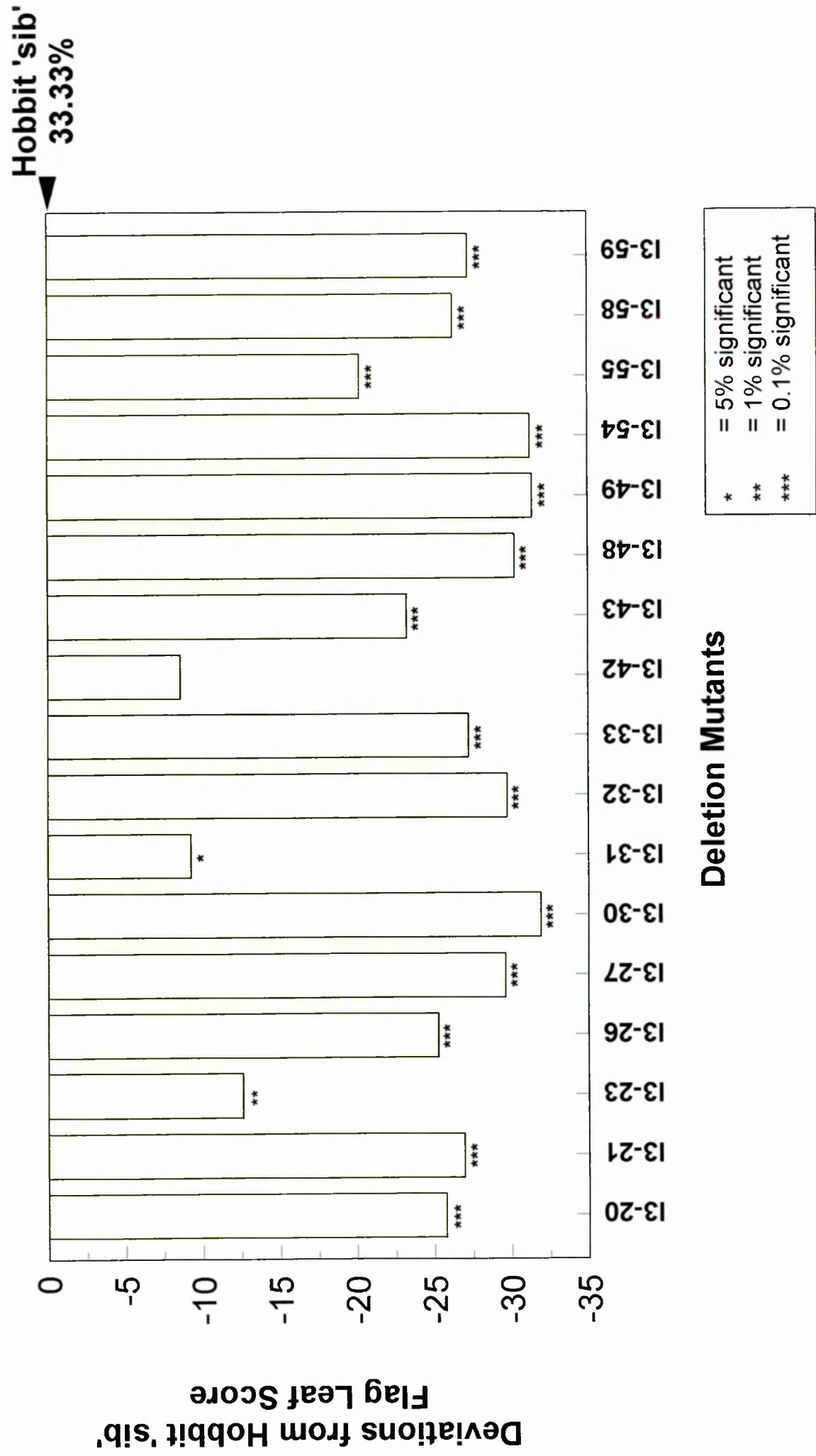


Fig 3.5: %flag leaf yellow rust infection at Morley Research Centre on 17 Hobbit 'sib' mutants, relative to Hobbit 'sib'. The single evaluation in 1995 showed a significant reduction in infection for 16 mutants.

trial, I3-42 was not significant. Further trials at this site would be necessary to confirm these results. The results show good correlation with the PBI Cambridge 3 year means for those lines tested (Correlation coefficient (r) = 0.788; p = <0.01). There is good correlation between the Cambridge and Morley 1994/1995 results once I3-20 and the off-type I3-23 are removed from the analysis (r = 0.482; p = 0.05). I3-20 was significantly more resistant at Morley than it was in any of the PBI Cambridge trials. It is possible that an incorrect seed stock was sown in the Morley trial. A repeat trial, ensuring the correct stock is sown at both sites would be necessary to clarify the result.

3.5.2 Brown Rust

There was a significant level of variation between the mutant lines and Hobbit 'sib' in both 1995 and 1996. These results are summarised as a two year average in Fig: 3.6. Three lines, I3-30, I3-32 and I3-48, showed consistent and significant brown rust resistance across both years.

There were three lines that were significantly more susceptible than the Hobbit 'sib' control in the 95/96 trial, namely I3-31, I3-33 and I3-37. In the 94/95 trial, both I3-31 and I3-33 were significantly more resistant than Hobbit 'sib', whilst I3-37 showed similar levels of disease to the control. It should be noted that I3-33 has a highly necrotic phenotype which may have affected the scoring accuracy. The susceptibility of I3-31 and I3-37 in the second year of trialling may have been due to a race-specific effect. A number of new brown rust races have emerged over the past few years (T.W. Hollins, pers. comms) and an increase in the levels of natural infection has been noted on Hobbit 'sib' at Morley Research Centre. The increase in susceptibility of certain mutant lines could therefore be due to the emergence of a new race. Alternatively, loss of resistance could be due to segregation. Both the susceptible lines have been subject to less selection and thus could still be segregating, although it is likely that segregation would have

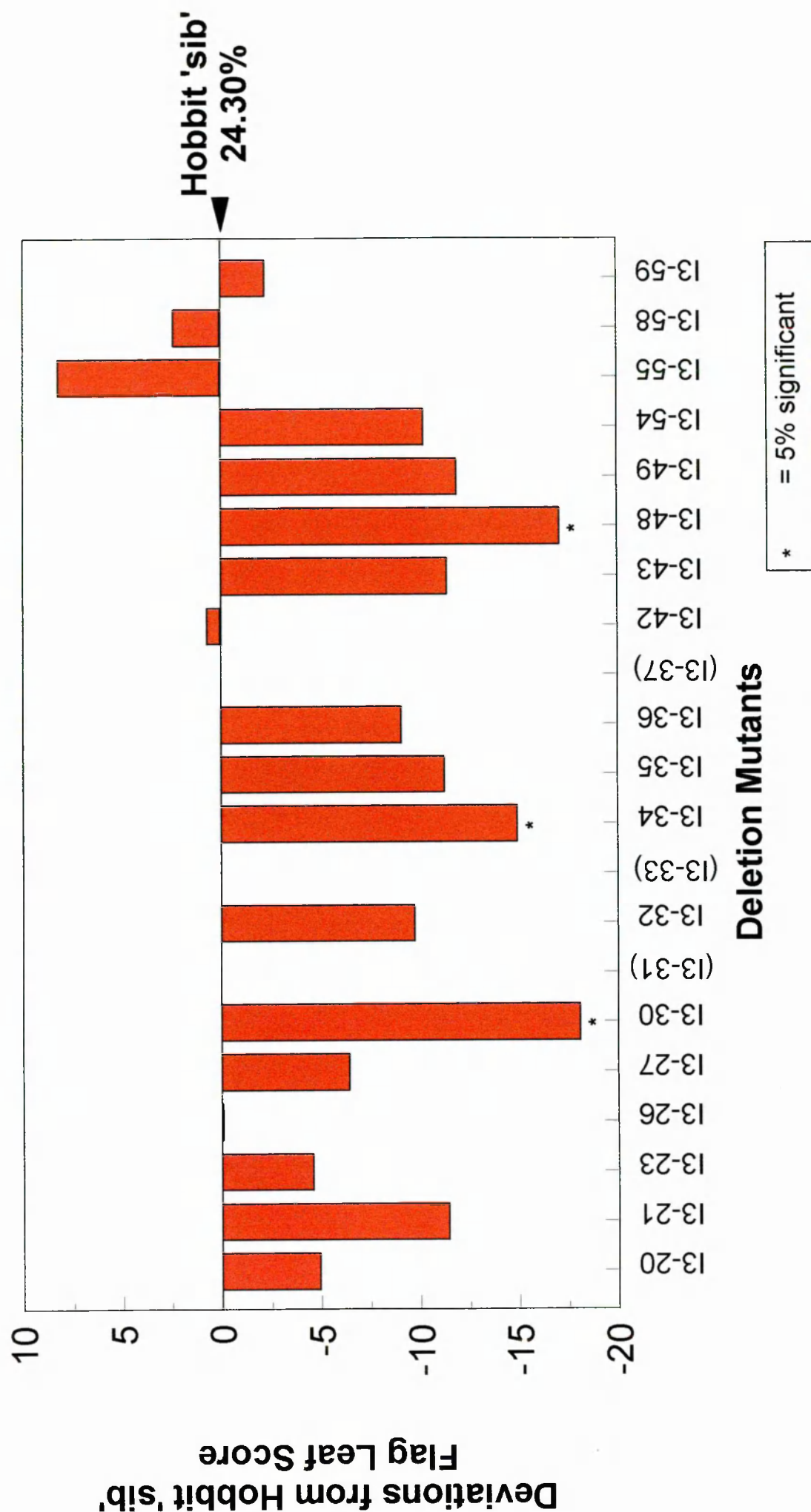


Fig 3.6: Average % flag leaf brown rust infection on 21 Hobbit 'sib' mutants, relative to Hobbit 'sib', showing a significant reduction in infection for 3 mutants. Each score represents a 2 year average infection. Scores for the mutant lines in brackets have not been included as results were inconsistent between years (see text).

been noted and scored as such.

The large variation between years in the three lines introduced a large error component to the two year analysis. Analysis of separate years had demonstrated a number of significant resistance effects. The three lines were therefore removed from the two year average analysis to reduce the 'trial/year' error component in the analysis of the remaining lines shown in Fig:3.6.

The original investigation on selected mutant lines indicated significant resistance to brown rust in the lines I3-27, I3-32, I3-43 I3-48 and I3-54 (Worland & Law, 1991). Of these 5 lines in the current study, only I3-48 showed consistently improved resistance. In addition, I3-30 and I3-34 showed increased resistance (not previously tested). At higher disease levels, as in 1996, I3-32 and I3-54 also exhibited increased resistance. This more intermediate level of resistance is probably indistinguishable at lower disease levels. Worland & Law observed that Hobbit 'sib' only reached a level of 13% flag leaf infection. The almost complete resistance of all the selected lines, including I3-32 and I3-54, in that work could well be a product of the low level of inoculum in that trial.

All of these results are based on two years trials. To confirm these effects, it would be necessary to carry out further field trials, particularly to resolve the causes of some of the more varied results discussed above. Glasshouse testing of I3-48, as discussed in Chapter 5, gave flag leaf infection levels of 2 - 15%, whereas Hobbit 'sib' was typically 40 - 60% infected. This further supports the field results on this line.

All three brown rust resistant lines, I3-30, I3-32 and I3-48 also showed resistance to yellow rust.

3.5.3 Mildew

Two independent growth cabinet tests were carried out. However, as the eight lines being

tested were sown in non-randomised blocks, without any replicate structure, the tests are not statistically rigorous . However, the conditions of the controlled environment conditions should ensure maximum infection levels as the continuous circulation of air within the cabinet should have ensured continuous inoculation pressure and removed any area effects within the cabinet. These results should therefore give a good indication of mutant resistance to mildew.

The high level of inoculum was reflected in the two trial mean of 62.5% flag leaf infection apparent on the Hobbit 'sib' control (Fig: 3.7). It would appear that infection levels were generally lower in trial 2. However, in relative terms, patterns of resistance can be determined.

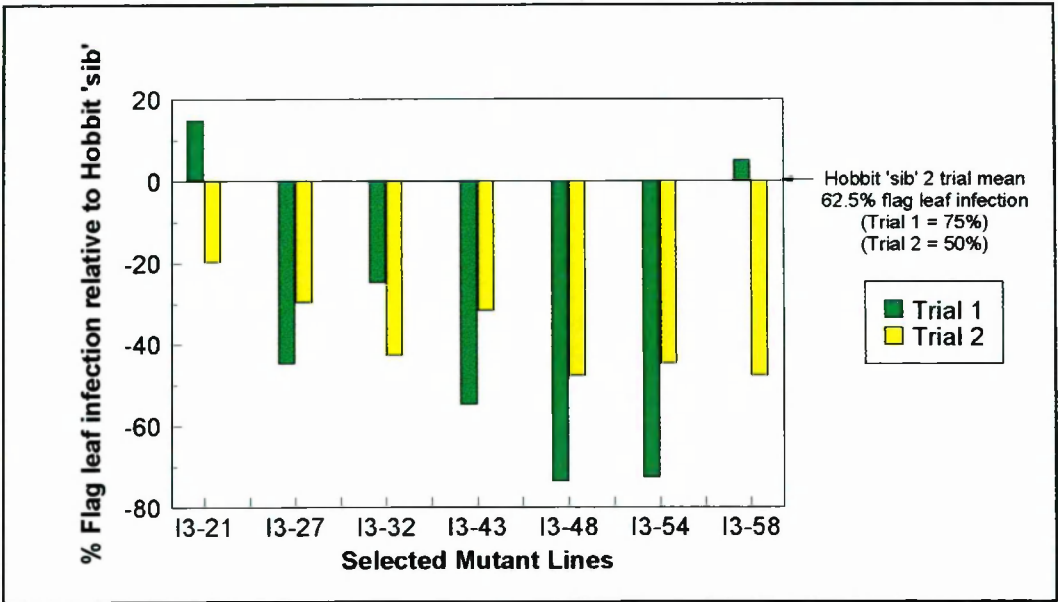


Fig 3.7: % Flag leaf infection of mildew on seven Hobbit 'sib' mutants for two separate trials, relative to the two trial mean % flag leaf infection of Hobbit 'sib'

As can be seen in Figure 3.7, I3-48 and I3-54 showed very high resistance to mildew infection. Intermediate levels of resistance were exhibited by I3-43, I3-27 and I3-32. I3-21 was extremely susceptible in Trial 1, although it only showed 30% infection in trial 2. No conclusions can be drawn on I3-58 as the results from the two trials were inconsistent. This may be due to the

introduction of a more aggressive race from the surrounding environment. Alternatively, the resistance may be affected by inoculum pressure, which was presumably higher in Trial 1, as indicated by the increased disease levels. It may be that the resistance to mildew infection is only effective up to a certain inoculum level, beyond which the resistance is overcome, giving a more susceptible phenotype.

When considering the inferences that can be made from the growth cabinet mildew tests, the high inoculum levels present under these conditions must be borne in mind. It is very unlikely that such high levels would be experienced in field trial conditions. Therefore, it is possible that those lines showing high susceptibility to mildew may not be quite so susceptible under field conditions. This may explain the lower resistance levels of certain selected mutants relative to the M10 field trial results (Worland & Law, 1991) discussed in section 3.1. The infection level of the Hobbit 'sib' control in the growth cabinet represents almost a ten-fold increase over that observed in the field. These conditions do, however, increase the confidence in the resistance of I3-48 and I3-54.

There is a correlated resistance between yellow rust and mildew for I3-48 and I3-54, and to some extent I3-27, I3-32 and I3-43, although the mildew resistance in the latter three lines is intermediate. Correlated resistance of yellow rust and powdery mildew has been noted previously (Pink *et al*, 1983) in Chinese Spring monosomic lines. It is possible that the correlated resistance of the mutants may be controlled by similar factors to those of Chinese Spring.

3.5.4 *Stagonospora nodorum*

As illustrated in Fig 3.8 and 3.9, none of the mutants exhibited significant resistance to *Stagonospora nodorum* over the two years of field trials. There were four mutant lines significantly more susceptible than Hobbit 'sib' at the flag leaf stage. These were I3-30, I3-33, I3-

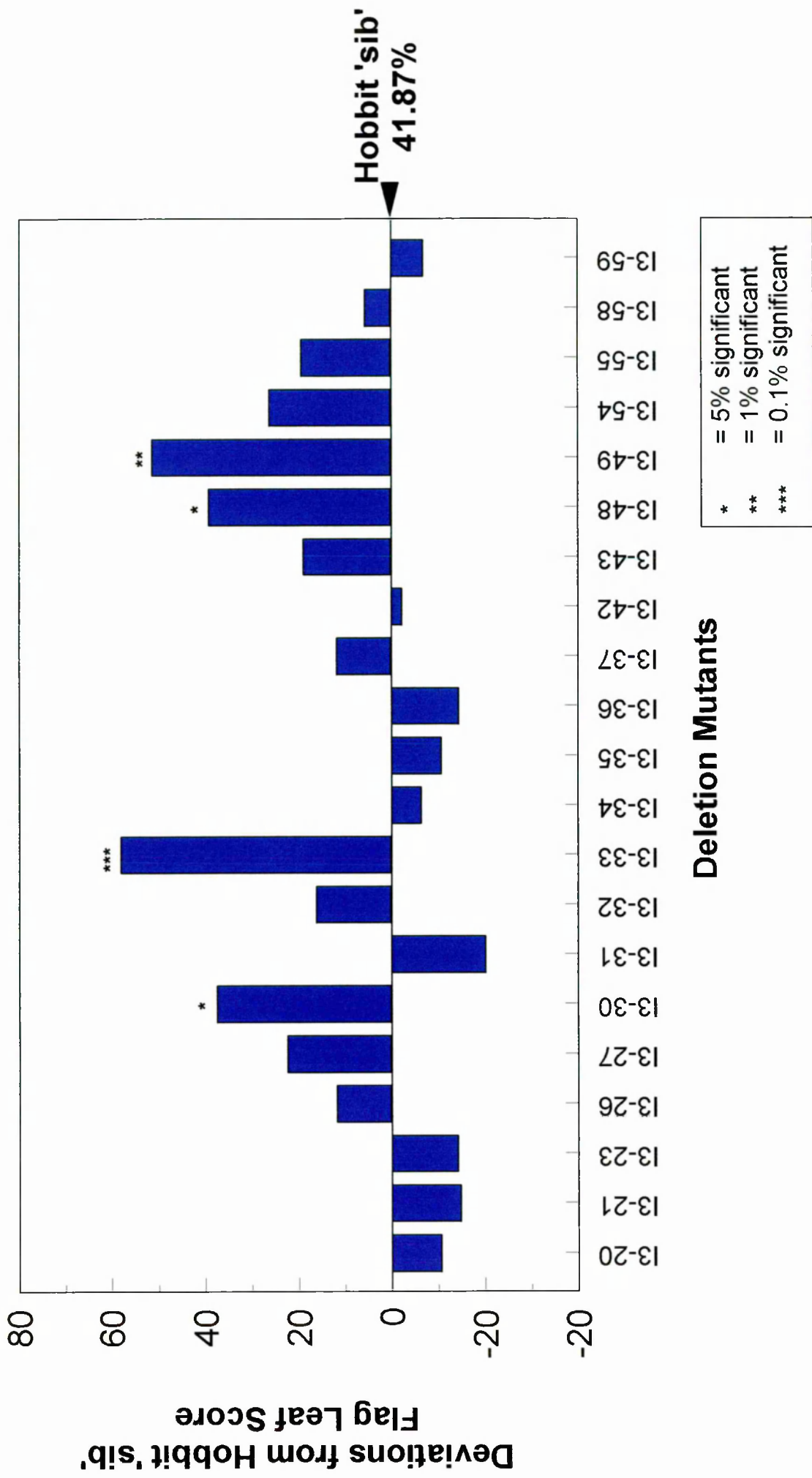


Fig 3.8: Average % flag leaf *Stagonospora nodorum* infection on 21 Hobbit 'sib' mutants, relative to Hobbit 'sib', showing a significant increase in infection for 4 mutants. No mutants have significantly increased resistance relative to Hobbit 'sib'. Each score represents a 2 year average infection.

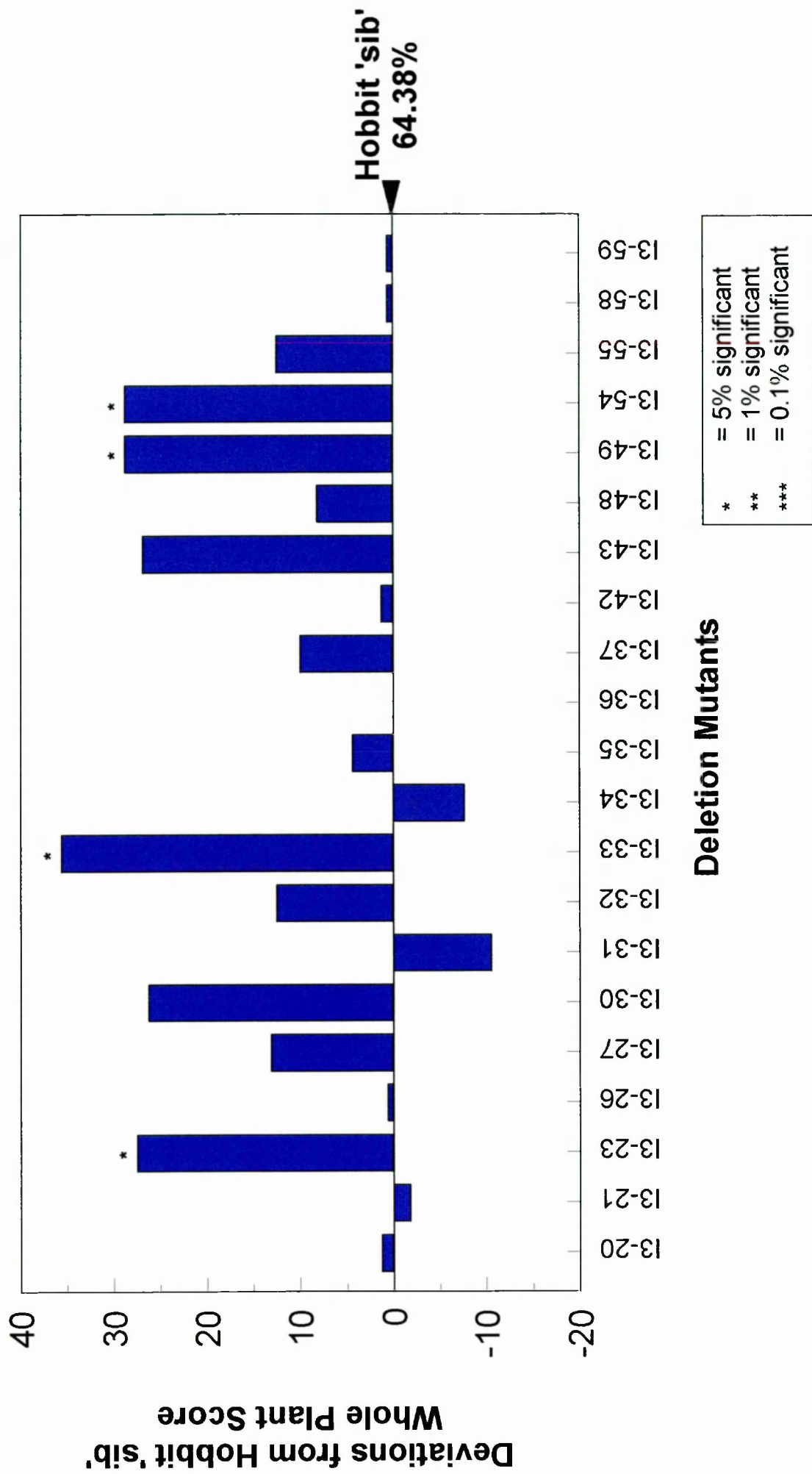


Fig 3.9: Average % whole plant *Stagonospora nodorum* infection on 21 Hobbit 'sib' mutants, relative to Hobbit 'sib', showing a significant increase in infection for 4 mutants. No mutants have significantly increased resistance relative to Hobbit 'sib'. Each score represents a 2 year average infection.

48 and I3-49. Two of these lines, I3-33 and I3-49, were also susceptible throughout the whole plant (Fig 3.8). In addition, I3-23 and I3-54 were susceptible, but not at the flag leaf stage.

The susceptibility of I3-30 can probably be attributed to a correlated effect of height. I3-30 was one of the shortest mutants tested. A positive correlation between height and disease resistance has been noted in wheat resistance to *S. nodorum* by Scott *et al* (1982) when studying F3 families of various wheat varietal crosses of differing heights. They proposed that this relationship could be due to either linkage of multiple loci to height alleles or a pleiotropic effect of height. It is likely that the pleiotropic effect is the cause of the susceptibility observed in the deletion mutants. *S. nodorum* is splash dispersed and is known to progress up through the plant. Therefore, in taller plants the inoculum has to undergo more rounds of dispersal and travel further in order to inoculate the upper leaves. The taller varieties will thus be less infected. Conversely, shorter lines will be more infected as they are closer to the source of infection. The artificial inoculation of the lines with overhead spraying in these trials may have negated this effect. However, it is also possible that the micro-environment surrounding shorter lines will be more favourable to disease multiplication as it is likely to be more humid, favouring disease development.

There are still some effects that cannot be explained by height. I3-48 and I3-49 were significantly more susceptible than Hobbit 'sib', but had heights close to the canopy average. It is possible that in these cases, resistance complexes have been deleted leading to higher susceptibility.

The highly susceptible phenotype of I3-33 can probably be attributed to the necrotic nature of the line. The saprophytic nature of *S. nodorum* may enable the pathogen to utilise the dying tissue of the host line to the full, hence the high level of infection.

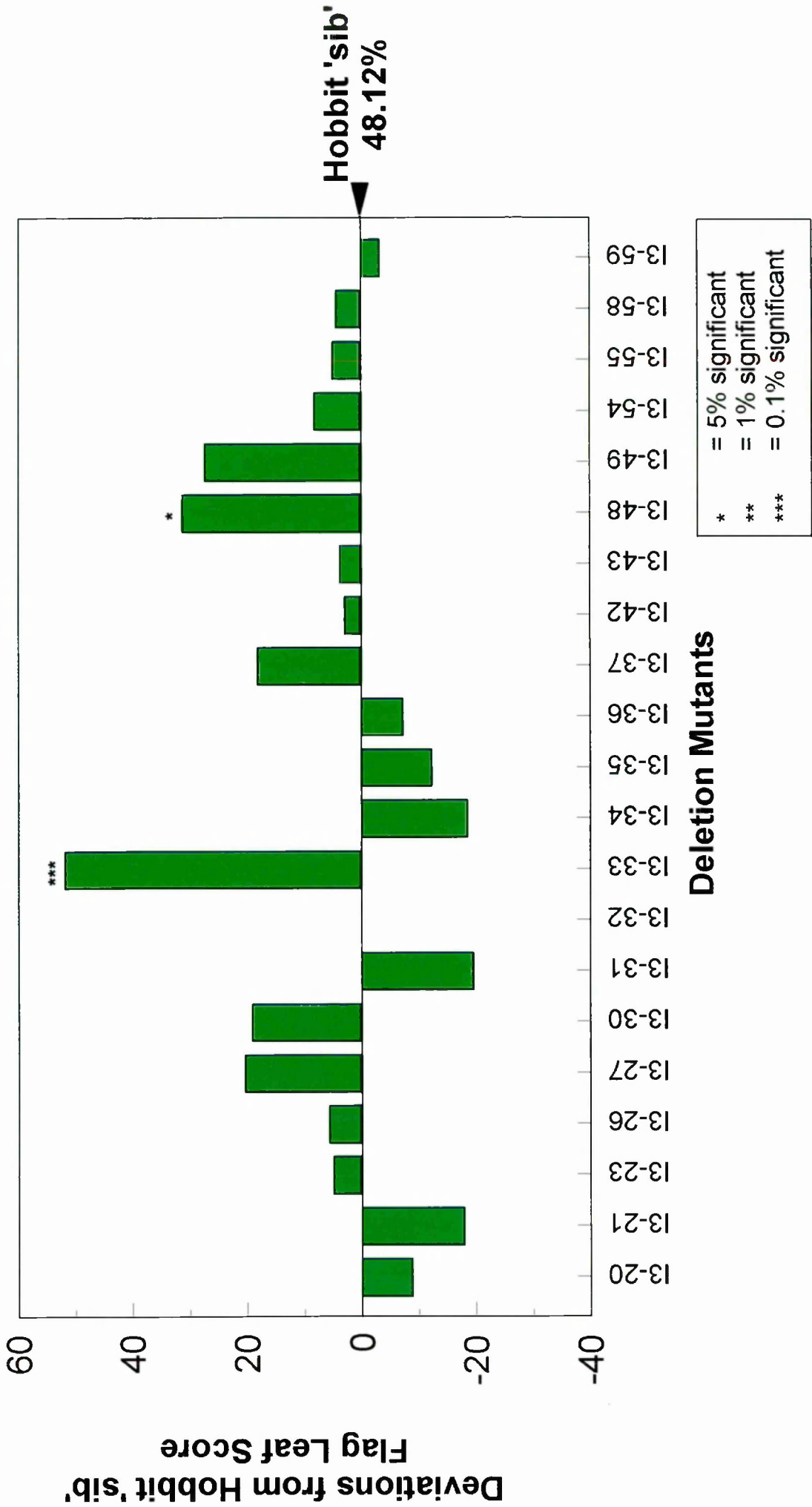


Fig 3.10: Average % flag leaf *Septoria tritici* infection on 21 Hobbit 'sib' mutants, relative to Hobbit 'sib', showing a significant increase in infection for 2 mutants. No mutants have significantly increased resistance relative to Hobbit 'sib'. Each score represents a 2 year average infection.

No explanation can be given for the increased susceptibility of I3-23 and I3-54 at the whole plant stage. The susceptibility may be due to loss of resistance or a morphological change caused by a deletion.

3.5.5 Septoria tritici

As with the *S. nodorum* data, there was no consistently significant resistance to *S. tritici* by any deletion mutant over the two years of field trials (Fig 3.10). I3-33 and I3-48 were significantly more susceptible than Hobbit 'sib'. The susceptibility of I3-48 to both *Stagonospora nodorum* and *Septoria tritici* suggests that some morphological character or component of resistance has been altered in the line, making it more susceptible.

3.6 Summary

Table 3.2 shows a summary of all trial data means for yellow rust, brown rust and mildew. Seventeen lines have been highlighted where they have shown consistent resistance (5% significance level or higher) to these three diseases. *S. nodorum* and *S. tritici* means have not been included as they showed no consistent and significant resistance relative to Hobbit 'sib'.

Of the 21 mutant lines tested, 12 showed consistently improved resistance to yellow rust over the three years relative to Hobbit 'sib'. I3-33, I3-34, I3-35, I3-36 and I3-37 have not been included in these twelve lines as they were not included in the 94/95 trial and as such only have 2 years data. However, all five lines were resistant in the two years they were tested, but would need further testing to confirm these results.

Three of these lines were also consistently resistant to brown rust, namely I3-30, I3-34 and I3-48. The two mutant lines showing high levels of mildew resistance, I3-48 and I3-54, also showed consistent resistance to yellow rust. I3-48 therefore exhibited good resistance to all three

DELETION	% FLAG LEAF INFECTION				
	Yellow Rust		Brown Rust		Mildew
	3 Year Average		2 Year Average		Trial 1 Trial 2
I3-20	25.56		19.34		
I3-21	8.22	**	12.83		90.00 30.00
I3-23	21.17		19.67		
I3-26	11.89	**	24.17		
I3-27	4.62	***	17.84		30.00 20.00
I3-30	1.36	***	6.17	*	
I3-31	31.34		N/A		
I3-32	8.94	**	14.50		50.00 7.00
I3-33	13.41	**	N/A		
I3-34	11.91	**	9.34	*	
I3-35	10.66	**	13.00		
I3-36	6.50	***	15.16		
I3-37	14.59	*	N/A		
I3-42	19.11		25.00		
I3-43	4.22	***	12.84		20.00 18.00
I3-48	2.40	***	7.16	*	1.00 2.00
I3-49	3.18	***	12.34		
I3-54	4.33	***	14.00		2.00 5.00
I3-55	14.57	*	32.50		
I3-58	10.78	**	26.67		80.00 2.00
I3-59	8.50	***	22.00		
Hobbit 'Sib'	30.55		24.30		75.00 50.00

Consistent Yellow Rust, Brown Rust and Mildew Resistance

Consistent Yellow Rust and Mildew Resistance

Consistent Yellow and Brown Rust Resistance

Consistent Yellow Rust Resistance Only

*** = Significant at 0.1% ** = Significant at 1% * = Significant at 5%

diseases. I3-30 and I3-54 showed good resistance to two diseases.

As can be seen from Table 3.2, the disease resistance of most of the deletion mutants tended to be restricted to yellow rust. A small number of mutants were also resistant to brown rust and/or mildew. Interestingly, of the original five mutant lines selected for further disease testing at M7 - M10 (Worland *et al*, unpublished), all five have remained resistant to yellow rust. However, only one of these lines has shown consistent brown rust resistance; only two of the original five have shown consistent resistance to mildew. It should be noted that in the original investigation of the five lines, disease pressure for both brown rust and mildew was considerably less than in the current tests, where there was a three fold increase in the Hobbit 'sib' disease level for brown rust, and an eight fold increase in mildew disease infection. It is therefore feasible that at low disease levels, there was insufficient disease pressure to infect the five lines significantly. At higher levels, as present in the latest trials, intermediate levels of disease developed on these lines.

No resistance to the facultative pathogens, *Septoria tritici* and *Stagonospora nodorum*, was demonstrated. Possible explanations for these results are discussed in Chapter 7.

In conclusion, the Hobbit 'sib' mutant series has now been extensively investigated for five of the more common wheat fungal diseases. These mutant lines provide a good potential source of resistance to a number of diseases, and demonstrate the effectiveness of irradiation in improving disease resistance.

CHAPTER 4

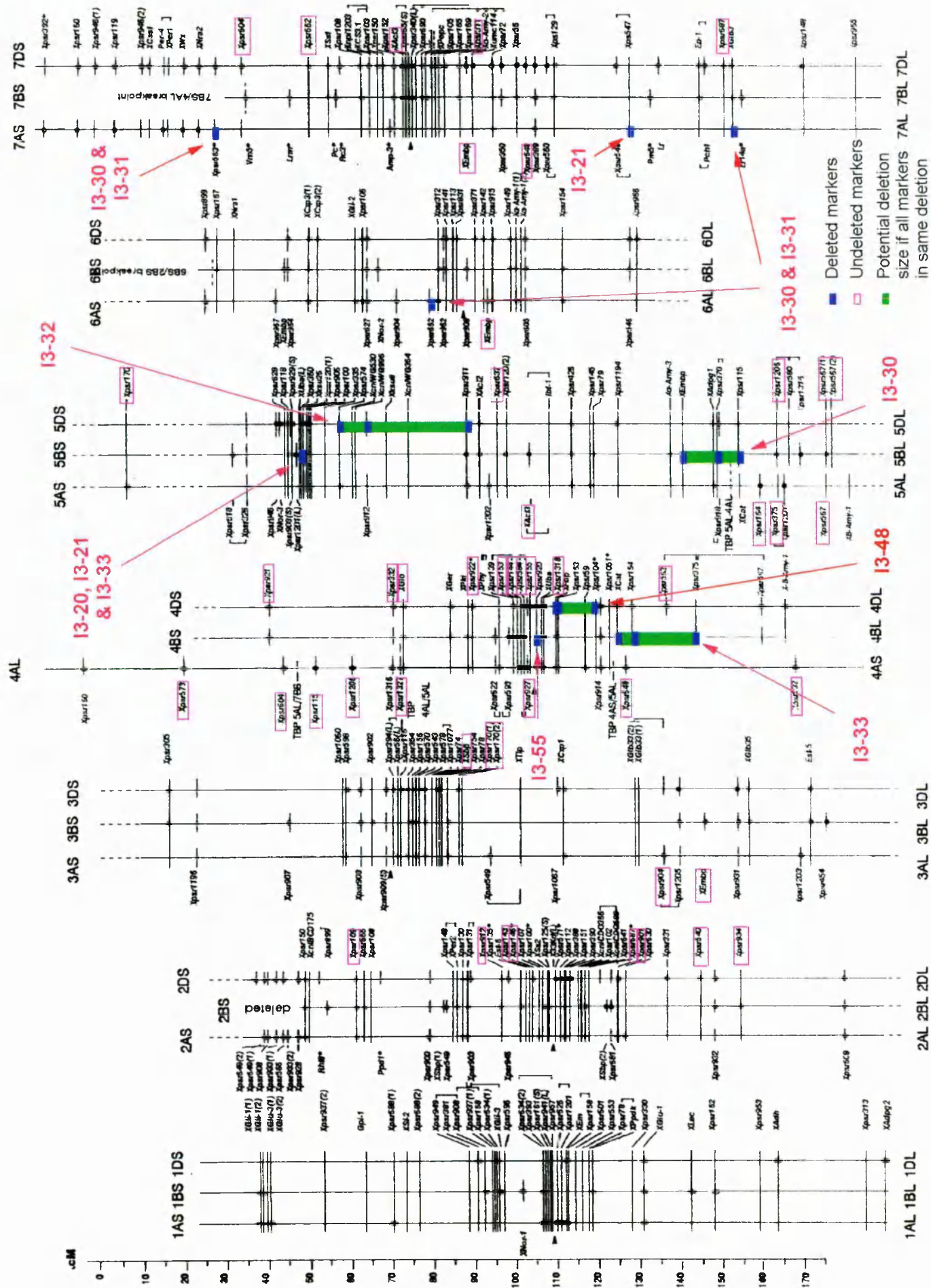
MAPPING OF DELETED SEQUENCES IN HOBBIT 'SIB' MUTANTS

4.1 Introduction

The previous chapter has demonstrated that a number of mutants showed increased resistance relative to Hobbit 'sib' for several diseases including yellow rust, brown rust and mildew. It has been hypothesized by Worland & Law (1991) that the deletion mutants have lost genes for susceptibility, thus conferring a higher level of resistance. In order to test this hypothesis, it is necessary to identify the locations of these deletions. If the deletions are located in regions carrying susceptibility alleles, it should be possible to demonstrate co-segregation of the deletions with resistance. This can be tested if the deleted region is marked so that segregation of the marker with resistance/susceptibility can readily be followed. Markers may also be the first step towards identifying the susceptibility alleles, which may lead to cloning these genes as well as providing a means of tracking the resistance conferred by the deletions through breeding programmes.

The aim of the work described in this chapter was to identify specific markers for the deletions hypothesized to be present in the mutant lines. Two approaches were taken. The first was to use existing Restriction Fragment Length Polymorphism (RFLP) markers to identify map positions of the deletions. The markers were obtained from those used in establishing the wheat genomic map being constructed at the Cambridge Laboratory, John Innes Centre (Gale *et al*, 1993). The second method, known as Representational Difference Analysis (RDA), was used as a more target specific approach to generate markers for certain mutants. The technique used subtractive hybridisation and kinetic enrichment to isolate restriction endonuclease fragments present in Hobbit 'sib' but not in the mutant. Theoretically, the isolated fragments should represent areas of the parental genome deleted in the mutants under investigation. The techniques are described further in the relevant sections.

Figure 4.1: Map locations of RFLP markers identifying deletions in Hobbit 'sib' mutants. The Xpsr markers are shown on the Cambridge Laboratory (JIC) Wheat Genome Map. Deletion points are shown in blue, with green banding between deleted markers, indicating the potential size of the deletion if all adjacent markers form part of the same deletion. Additional markers used in screening but not identifying a deletion are circled pink.



4.2 RFLP Screening

A general screening for deletions was carried out using RFLP markers supplied by the RFLP club, John Innes Centre. The purpose of the screening was to identify deletions and their approximate location. A total of 57 markers, covering 6 chromosome groups, from group 2 to group 7, were used in the screening. Since previous monosomic analysis of the mutants had shown that there were resistance effects determined by the group 4 and 5 chromosomes, initial screening was concentrated on these two groups. The map positions of identified deletions are shown in Figure 4.1. Estimates of deletion length are based upon the assumption that adjacent deleted markers are part of the same deletion.

4.2.1 Chromosome Group 4

Using a total of 24 markers, spanning the group 4 chromosomes, three deletions were found.

I3-55 showed a deletion on 4BL at the locus marked by Xpsr 920. This deletion appears to be relatively small. The nearest undeleted proximal marker to Xpsr 920 is only 1 cM away whereas the nearest undeleted distal marker is a maximum of 5 cM from the deletion. This would make the overall deletion no more than 6 cM in length.

I3-33 also carries a deletion on 4BL. Three markers were shown to be deleted in I3-33. However, due to the paucity of markers towards the terminal end of 4BL, it is uncertain whether the deleted markers involve one or several deletions. The proximal end of the deletion terminates before the marker Xpsr 104 which is 5 cM proximal to the nearest deleted marker (XCat). The most distal deleted marker of 4BL is Xpsr 375. However, as this is the most distal mapping marker on this chromosome, the distal endpoint of the deletion can not be determined. If this was near to Xpsr 375 then the deletion would be a minimum of 24 cM in length. Further markers are

Figure 4.2: The use of RFLP probe Xpsr 104 to detect a deletion on chromosome 4DL in mutant I3-48. The autoradiograph of the Southern blot shows DraI digested DNAs of 21 mutant lines with Hobbit 'sib', Bezostaya and Chinese Spring controls, with a λ /HindIII digested size marker.

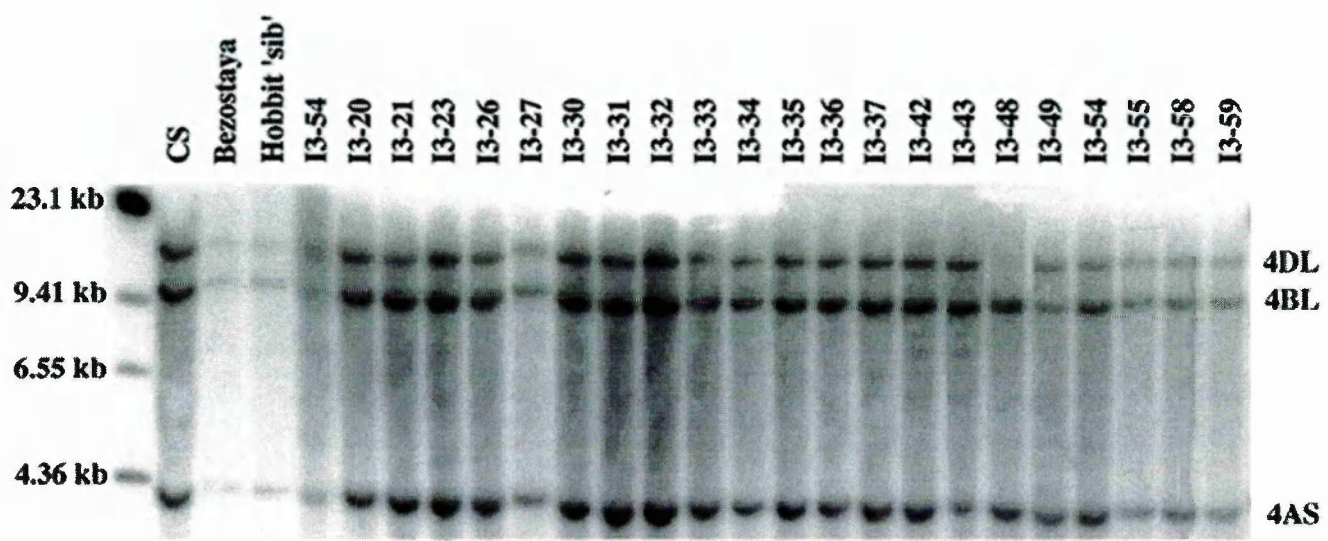
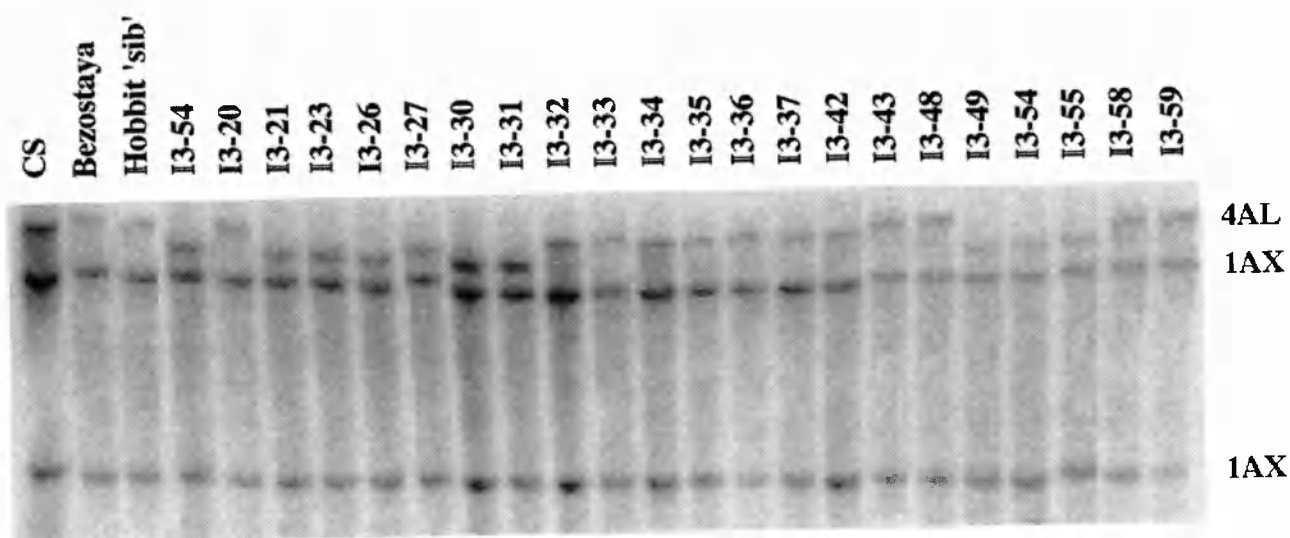


Figure 4.3: The use of RFLP probe Xpsr 1327 to detect a polymorphism on chromosome 4AL. Nine mutants show the same polymorphism, indicated by the reduced size 4AL band, relative to Hobbit 'sib'. The autoradiograph of the Southern blot shows EcoRV digested DNAs of 21 mutant lines with Hobbit 'sib', Bezostaya and Chinese Spring controls.



needed in this region before the actual length of the deletion can be determined with any accuracy.

I3-48 lacks 3 loci on chromosome 4DL: XFbp, Xpsr 163 and Xpsr 104 (Figure 4.2). The nearest marker known to be present proximal to the deletion is Xpsr 1318, which is 3 cM away from XFbp. The nearest distal marker is XCat, approximately 9 cM from the distal deleted marker. The maximum length of the deletion is therefore 22 cM.

Probe Xpsr 1327: RFLP analysis of the mutant population with Xpsr 1327, which maps on 4AL, close to the site of the primitive translocation between 4AL and 5AL showed a single polymorphism between two groups of mutants. Mutant lines I3-21, I3-23, I3-26, I3-27, I3-30, I3-31, I3-49, I3-54 and I3-55 all showed this polymorphism relative to Hobbit sib and the remainder of the mutants (Fig 4.3). The polymorphism may represent a common deletion adjacent to the Xpsr 1327 locus in the 9 mutant lines, or may be a product of the 4AL/5AL translocation.

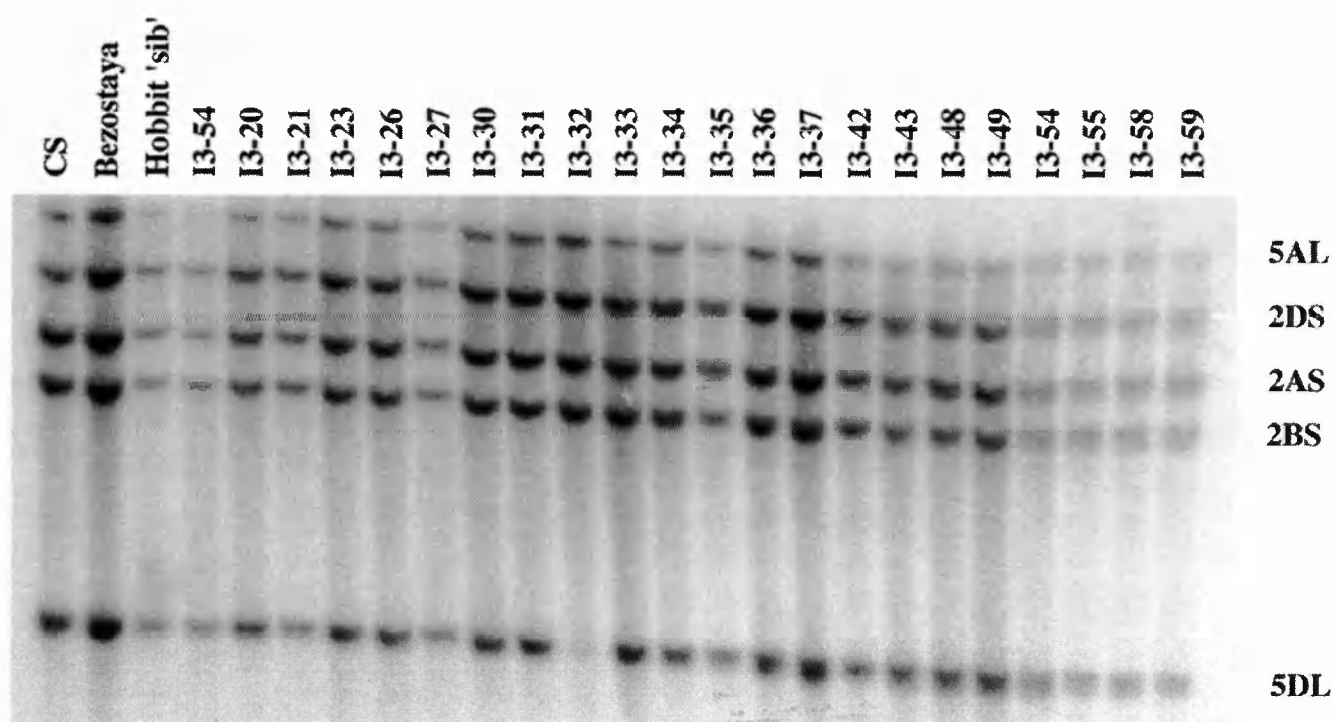
4.2.2 Group 5 Chromosomes

Using a total of 17 markers, spanning the group 5 chromosomes, three deletions in five mutant lines were identified.

A deletion common to lines **I3-20**, **I3-21** and **I3-33** was found at the Xpsr 360 locus on 5BL. The proximal limit of the deletion was not determined. The distal limit is at a point proximal to Xpsr 574. However, Xpsr 360 and Xpsr 574 are 14 cM apart, and there are five untested markers between them, mostly clustered close to Xpsr 360. As the three deletion lines exhibited medium levels of resistance only, the deletion was not investigated further.

I3-30 was identified as lacking three loci on 5BL, namely XEmbp, Xpsr 370 and Xpsr 115. Since Xpsr 1206 was present, the distal limit of the deletion must lie between it and Xpsr 115. No marker between XAcl3 and XEmbp was tested so an exact proximal limit is unknown.

Figure 4.4: The use of RFLP probe Xpsr 912 to detect a deletion on chromosome 5DL in mutant I3-32. The autoradiograph of the Southern blot shows EcoRV digested DNAs of 21 mutant lines with Hobbit 'sib', Bezostaya and Chinese Spring controls.



I3-32 lacked three markers on the long arm of 5D. The nearest marker indicating the maximum proximal limit of the deletion was Xpsr 360; 14 cM from Xpsr 574. The distal limit was at a point before Xpsr 637; which is approximately 6 cM distal to the deleted Xpsr 911 locus. Thus the deletion could have a maximum size of 52 cM. The deleted Xpsr 912 locus is shown in Figure 4.4.

4.2.3 Group 7 Chromosomes

Ten probes, distributed along the group 7 chromosomes, detected 3 deletions. Each deletion is identified by a single missing locus. No attempt was made to investigate the size of the deletions.

Two deletions were found in **I3-30** and **I3-31**; one at a locus marked by XGlb3 on 7AL, the other by Xpsr 563 on 7AS. One deletion in **I3-21** was mapped to Xpsr 547 on chromosome 7AL.

A possible deletion in **I3-23** has also been mapped to Xpsr 687 on 7AL just above the I3-30 and I3-31 deletion. However, doubts over purity of I3-23 have been expressed by A. J. Worland (pers. comm) where previous phenotypic observations and field data suggested that the line was an 'off type'. Indeed, several polymorphic loci have been noted: Xpsr 580, XEmbp on 5DL; Xpsr 150 on 7DL, suggesting the line was an outcross. No polymorphism has been observed with any of the other mutants investigated, other than at the Xpsr 1327 locus on 4AL.

4.2.4 Other Chromosome Groups

Eight markers were tested on the group 2 chromosomes and four markers mapping to the group 3 chromosomes, but no deletions were detected. One marker, Xpsr 662, also mapped to 6AS, in addition to group 3. **I3-30** and **I3-31** contained a deletion on 6AS at the Xpsr 662 locus.

4.3 Representational Difference Analysis

Although a number of deletions had been identified using general RFLP screening, deletions in certain mutant lines having high levels of disease resistance, such as I3-54 and I3-27, were not detected. For example, field results had indicated that the mutant line I3-54 showed good resistance to yellow rust and mildew, and according to monosomic analysis of this line, the resistance was determined by 4B. However, a comprehensive screening of the group 4 chromosomes with the available RFLP markers had not identified a deletion. It was therefore decided that a more focused approach to identify deletions within a specific line was needed. Representational Difference Analysis (RDA) was chosen as it should theoretically allow the direct isolation of sequences corresponding to the deleted sequences in the mutant. Such sequences could then be screened against the deletion mutant series to check for positive clones, i.e. clones that identified deletions in the mutant lines.

Representational Difference Analysis, as described by Lisitsyn *et al* (1993), was proposed as "a general method for finding small differences between the sequences of two DNA populations". Briefly summarised, the technique involves restriction digestion of two DNA populations, one of which contains the target sequences of interest. The DNA is mixed, denatured and then allowed to reassociate. Sequences in common between the two populations anneal to each other whilst sequences unique to one population (the target sequences) can only self-reanneal. An excess of the DNA population lacking the target sequences ensures that common sequences in the target population are more likely to cross-anneal rather than self-reanneal. Ligation of specific PCR adaptors onto the target DNA only ensures that only self-reannealed target sequences are PCR amplified. Thus, an enrichment of target sequences in the DNA population is achieved. Repeating this cycle increases the rate of enrichment, as the proportion

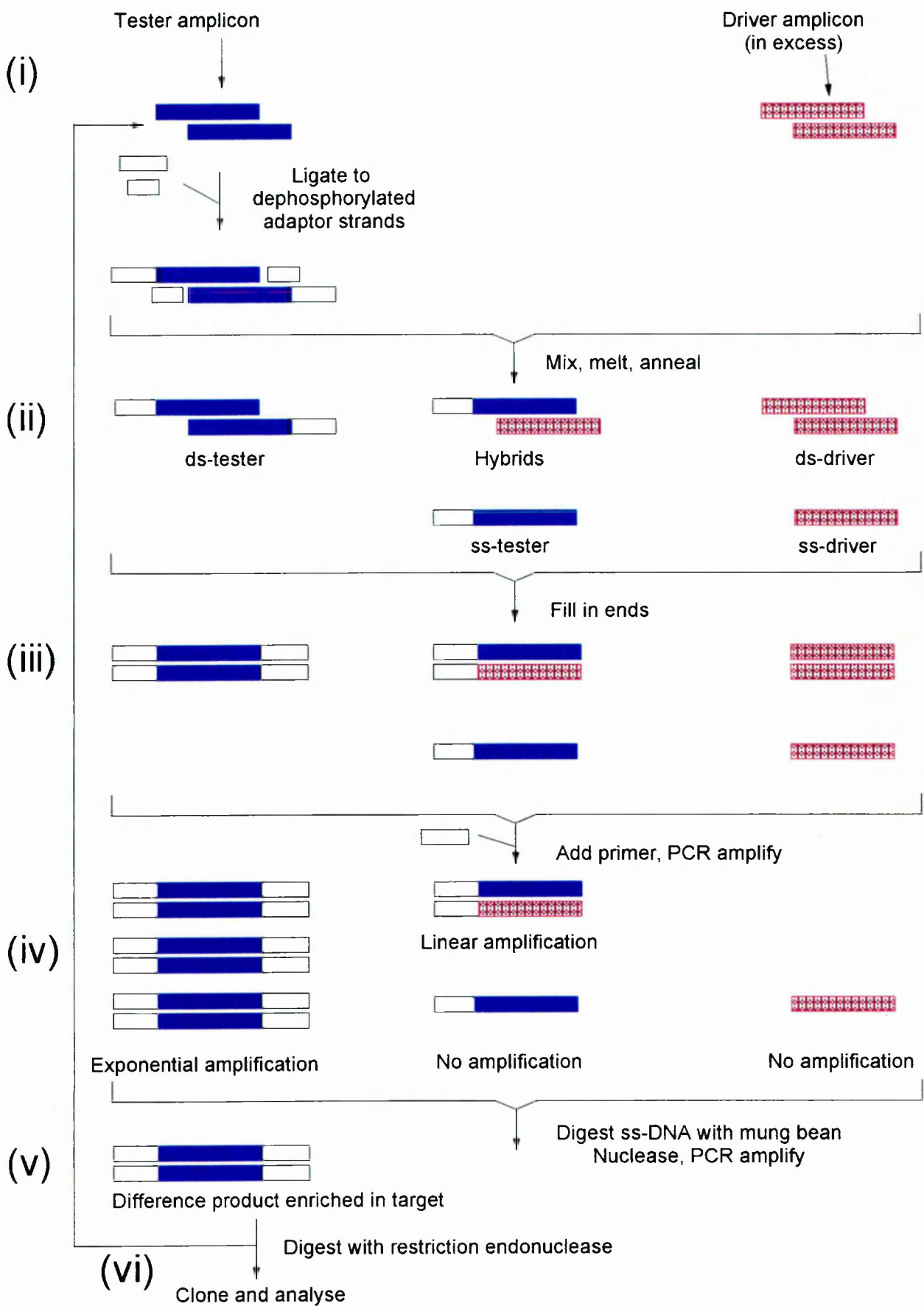
of 'common' sequences in the population drops.

The method was based upon subtractive hybridisation techniques described previously (Kunkel *et al*, 1985; Nussbaum *et al*, 1987) to clone probes for human X-chromosome deletions associated with Duchenne muscular dystrophy and choroideremia respectively. Subtractive hybridisation has also been used to clone DNA sequences corresponding to deletions in plants systems, as reviewed by Chasan (1992).

The basic approach of subtractive hybridisation is to mix wild-type DNA (known as tester DNA as it contains the target sequences) with excess DNA from a deletion mutant (known as driver DNA) that lacks the desired sequences, denature the DNAs and allow them to reassociate. Reannealed molecules in which one or both the strands contain the DNA from the deletion mutant are then removed. The remaining DNA after this "subtraction" should be enriched in the deleted sequences from the tester. Although these single step subtractions have been used successfully, the enrichment of deletion specific sequences is no more than the ratio of the two types of DNA. A greater degree of enrichment has been achieved by carrying out multiple subtractions. This technique has been used by Sun *et al* (1992) to clone the gibberellin biosynthesis gene, GA1, in *Arabidopsis*. Following several rounds of subtractive hybridisation, the small amount of product remaining was PCR amplified before cloning the DNA.

RDA achieves enrichment beyond the capabilities of multiple subtractive hybridisation by coupling subtractive hybridisation with DNA amplification to achieve "kinetic" enrichment. The initial stage of the process involved making a representation of the Hobbit 'sib' (tester) and deletion mutant (driver) DNA populations by cleaving the DNA with an infrequently cutting, methylation sensitive restriction enzyme and ligating oligonucleic adaptors prior to PCR amplification to form "amplicons" (Fig 4.5 (i)). The use of the methylation sensitive enzyme acts

Fig 4.5: Schematic protocol for one round of representational difference analysis, used to isolate deletion specific clones, as described in Chapter 4.3.1. Alternate primer sets, as detailed in Chapter 2.2.14, are used with each successive repetition of the cycle.



to reduce the complexity of the genomic DNA, as only non-methylated segments of the genome are 'represented', thus allowing greater completeness of subsequent subtractive enrichment stages and hence achieving more efficient kinetic enrichment. Once tester and driver amplicons are made, adaptors are removed by enzymatic cleavage, and only tester fragments are ligated to new adaptors at their 5' ends. After melting and reannealing tester DNA in the presence of excess driver amplicon (Fig. 4.5 (ii)), DNA amplification is used for selective enrichment of double-stranded tester. Only self-reannealed tester molecules have 5' adaptors at the end of each duplex DNA and thus can be filled in at both 3' ends (Fig 4.5 (iii)). Hence, only self-reannealed tester can subsequently be amplified by PCR at an exponential rate (Fig. 4.5 (iv)). The presence of excess driver DNA in the reannealing phase acted as a competitive inhibitor for the self-reannealing of tester DNA fragments common to the driver. Target DNA, which occurs only in the tester, is thus enriched relative to other tester DNA after amplification (Fig. 4.5 (v)). Further enrichment (Fig 4.5 (vi)) is achieved by cleaving the PCR products and adding new PCR adaptors to the 5' ends before repeating the hybridisation-amplification step.

After two rounds of hybridisation-amplification, the PCR products are cloned into puc19 vectors and analysed using deletion mutant filters. Prior to screening, all clones are screened for the presence of repeated sequences. Clones that are repeats are screened out by probing small volumes of each clone, dotted onto nylon membrane, with genomic wheat DNA from the variety 'Chinese Spring'. Repeat sequences are identified as they give high radioactive signals, whereas single copy clones do not hybridise to the genomic DNA.

4.3.2 Screening of RDA Clones

RDA was used to identify deletions in mutants I3-54 and I3-27. Clones extracted after two rounds of the experiment were screened against the deletion mutant blots used for the RFLP

Table 4.1: Results of the Representational Difference Analysis method, used to isolate clones specific to deletions. A number of clones were isolated, some of which were apparently identical (shown in brackets), identifying deletions in several mutants. The clone size and the resulting band size (with the respective enzyme) in which the deletions were detected are detailed. Deletions were detected in several mutants for each driver source used. Interestingly, for both 4B/I3-54 backcross lines (AJW4/54 - JIC sourced; FIELD 4/54 - field sourced), no clones were isolated that detected deletions in the I3-54 mutant.

Clone no.	Driver Source	Clone size	Size of deleted band	Enzyme	Mutant line showing deletion
RDA 23	I3-54	580 bp	4.4 kb 5.1 kb	EcoRV EcoRV	I3-54, I3-21 I3-48
RDA 9	I3-54	450 bp	9.3 kb 2.5 kb	Dra I Dra I	I3-54, I3-21 I3-54
RDA 4 (RDA 18)	I3-54	450 bp	9.3 kb	Dra I	I3-54, I3-21
RDA 22	I3-54	680 bp	7.7 kb	Dra I	I3-54, I3-21
RDA X10 (RDA X16)	AJW 4/54	230 bp	7 kb	Dra I	AJW 4/54, I3-20, I3-21, I3-30, I3-31
RDA X7 (RDA X20)	AJW 4/54	510 bp	17.7 kb 4.1 kb	EcoRV EcoRV	AJW 4/54, I3-30, I3-31
RDA P25	FIELD 4/54	580 bp	5 kb	Dra I	I3-32
RDA P1	FIELD 4/54	420 bp	7.8 kb	EcoRV	I3-59
RDA Y12	I3-27	400 bp	4.9 kb 3.5 kb	Dra I Dra I	I3-26, I3-27 I3-32

screening (Table 4.1). Three driver genotypes were used to isolate deleted sequences in I3-54, namely I3-54 and two Adw4/I3-54 lines, 'AJW4/54' and 'Field 4/54' (one supplied by A.J. Worland, the other obtained from field trials), in which I3-54 was backcrossed to Hobbit 'sib' monosomic 4B (Adw4) and the progeny selfed to produce disomic plants. Deletions in I3-27 were targeted using I3-27 as a driver genotype. The choice of driver genotypes is explained in the relevant sub-sections.

RDA has been used successfully to identify deletions in both target lines, I3-54 and I3-27. Unexpectedly, in addition to the target mutants, deletions were identified in a number of additional mutants.

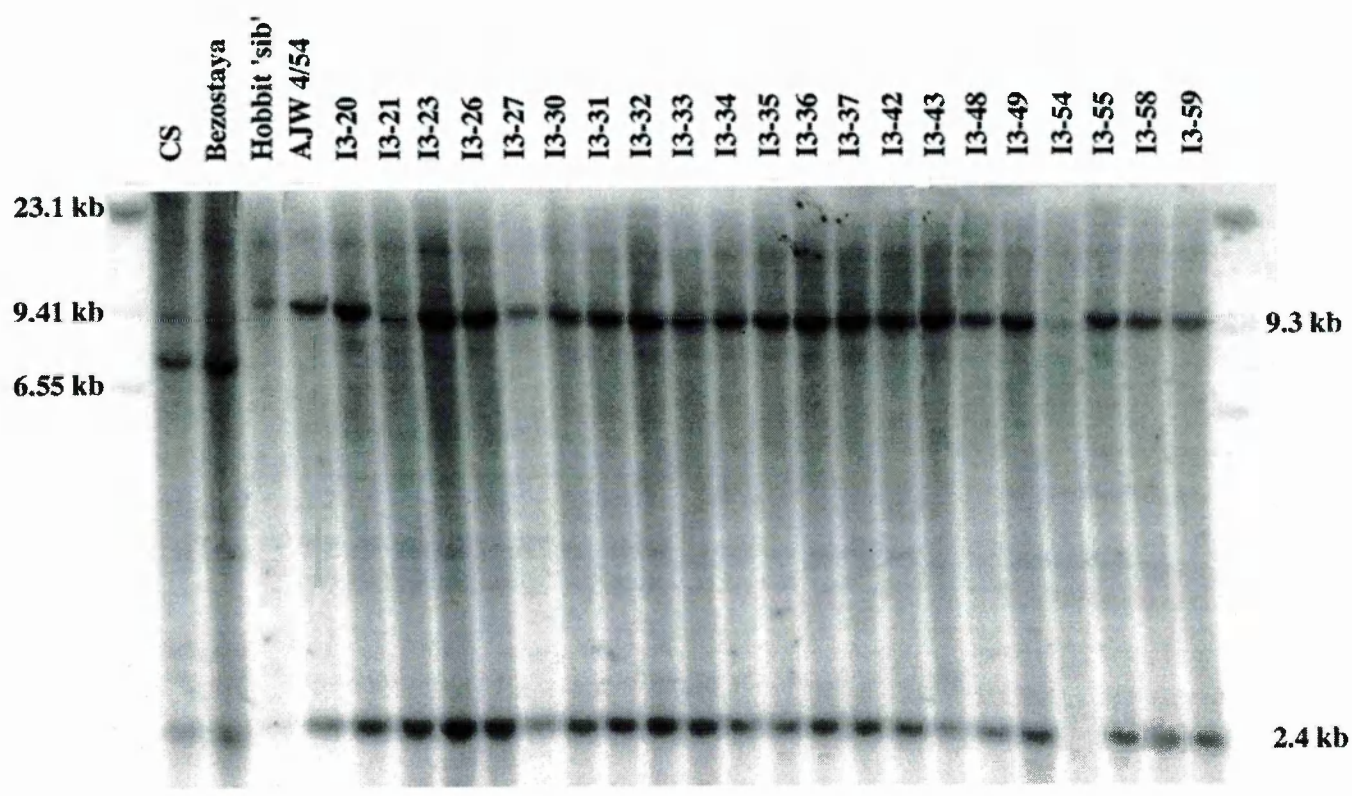
4.3.2.1 Deletion Mutant Line I3-54

I3-54 driver

As noted in section 4.3, no deletion had been identified in I3-54 using conventional RFLP screening. I3-54 was used as an initial driver genotype to isolate deletion specific clones. Of the 25 clones isolated after two rounds of the experiment, 17 non-repetitive clones were isolated. Five of these identified deletions in I3-54 (Table 4.1), although two of the clones (RDA 4 and RDA 18) had the same banding pattern, suggesting they were identical. All five clones were deleted in I3-21, in addition to the source deletion mutant. Furthermore, as well as identifying a deletion in I3-54 and I3-21, an additional band of 5.1 kb deleted in I3-48 was detected with clone RDA 23.

One clone, namely RDA 9 identified a shared deletion between both mutants in one band of 9.3 kb (Figure 4.6). However, an additional band (2.4 kb) was also deleted in I3-54 that was present in I3-21, suggesting hybridisation of RDA 9 to more than one chromosome and thus identifying an additional deletion in I3-54 that was not present in I3-21.

Figure 4.6: The use of RDA derived probe RDA9 to detect deletions in mutant I3-54 of the 9.3 kb and 2.4 kb bands, and a deletion in I3-21 of the 9.3 kb band. The autoradiograph of the Southern blot shows *Dra*I digested DNAs of 21 mutant lines with Hobbit 'sib', Bezostaya and Chinese Spring controls, with a λ /HindIII digested size marker.



'AJW4/54' driver

As a resistance effect in I3-54 had been identified on chromosome 4B using monosomic analysis, a Hobbit 'sib' monosomic 4B/I3-54 backcross line supplied by A.J. Worland that had undergone four backcrosses was selected as an alternative driver genotype. Theoretically, by continuously backcrossing this line to the 4B monosomic, the majority of the genotype should be Hobbit 'sib' type, whilst the 4B chromosome should be the same as I3-54. Therefore, only clones corresponding to deletions present on chromosome 4B of I3-54 should have been isolated when using this line.

Of the 23 clones obtained, twelve were successfully amplified, of which 7 were identified as single copy sequences and screened for specificity to the deletion mutants. Four clones indicated deletions in the backcross line, designated AJW 4/54 (Table 4.1), although RDA X10 and RDA X16 appear to have the same banding pattern, as do RDA X20 and RDA X7, suggesting two pairs of identical clones. As well as mapping to the source line, all four clones identified a deletion in I3-30 and I3-31. In addition, two of the clones, RDA X10 and RDA X16 also identified deletions in I3-20 and I3-21.

Surprisingly, none of the clones mapped to the original I3-54 line. This would suggest that the deletion present in AJW 4/54 did not originate from I3-54, indicating an original source of variation other than I3-54, possibly through a mutant line used in crossing being heterozygous. However, the variation is most likely to have originated in the Hobbit 'sib' monosomic used in the 4B backcross. During the construction of the monosomic, continuous backcrossing onto Hobbit 'sib' was carried out to reconstruct the Hobbit 'sib' background. However, during the backcrossing programme, the original Hobbit 'sib' recurrent parent, that was known to have been heterozygous and to be derived at a relatively early selfing generation in the development of

Hobbit 'sib', was switched for an alternative Hobbit 'sib' line that had undergone more selfing generations (A. J. Worland, pers. comm.). It is therefore feasible that there is variation in the background that has given rise to RDA clones extracted using the AJW 4/54 line.

One other possible source of variation giving rise to clones may result from polymorphism around the 4AL/5AL translocation site discussed in section 4.2.1. All the deletion lines except I3-20 identified by the RDA X clones are in the polymorphic group identified by Xpsr 1327. It is possible that the RDA X clones actually relate to sequence differences between Hobbit sib and the mutants due to translocation rather than deletions.

'Field 4/54' driver

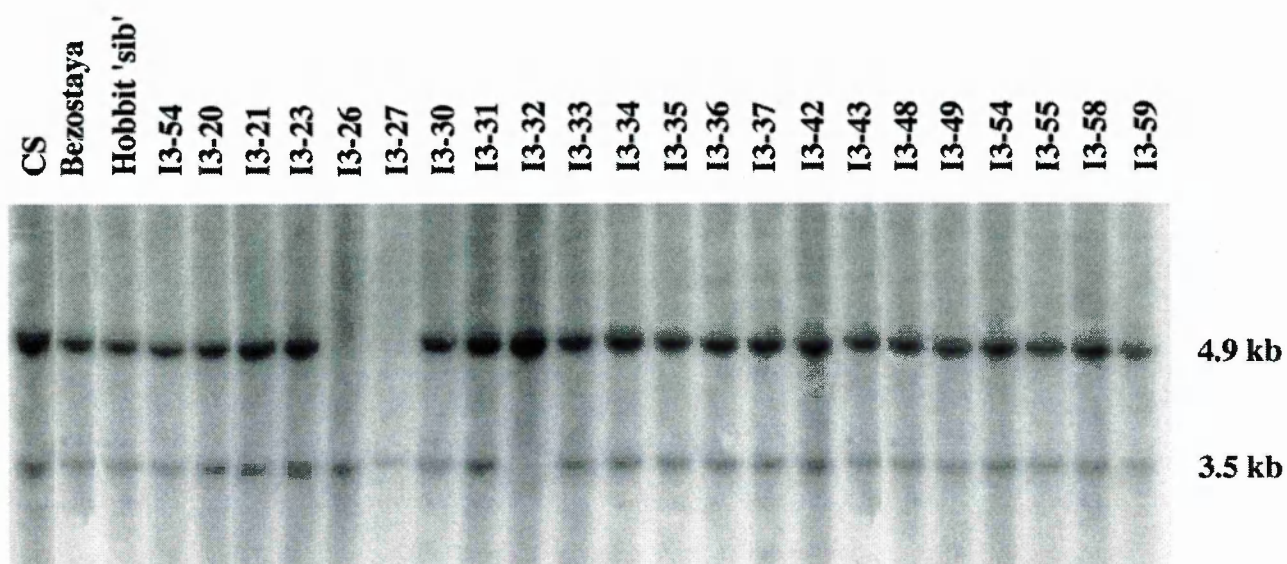
Due to the lack of clones identifying deletions in I3-54 with the first backcross line, a second alternative line, 'Field 4/54', that had been tested in field trials and showed similar levels of resistance to I3-54 was used as a driver genotype. A total of 106 clones were extracted, although 45 clones were shown to be identical. 16 clones of differing band sizes were selected and screened against the deletion mutant series. Two clones were shown to map to deletion mutants. Clone P25 showed a deletion in mutant I3-32. RDA P1 showed a deletion in mutant I3-59 (Table 4.1). No clones mapping to I3-54 or the AJW 4/54 backcross line were found. This may be due to heterozygosity or variation due to outcrossing in the field-derived backcross line.

4.3.2.2 Deletion Mutant Line I3-27

RFLP screening had failed to identify deletions in this mutant line. As the line had demonstrated a good level of yellow rust resistance, I3-27 was selected as suitable target in which to identify new deletions.

Of the 24 clones originally isolated after two rounds of RDA, 17 single copy sequences were used to screen the Hobbit 'sib' mutants for deletions. One clone, RDA Y12, mapped to I3-27

Figure 4.7: The use of RDA derived probe RDA Y12 to detect deletions in mutants I3-26 and I3-27 of the 4.9 kb and an additional deletion in I3-32 of the 3.5 kb band. The autoradiograph of the Southern blot shows DraI digested DNAs of 21 mutant lines with Hobbit 'sib', Bezostaya and Chinese Spring controls.



(Figure 4.7). The clone also co-mapped to I3-26 and I3-32. I3-26 had the same deleted 4.9 kb band as I3-27, whilst I3-32 had an alternative deletion of a 3.5 kb band.

4.4 Mapping of RDA Clones

The RDA clones were mapped using nullisomic-tetrasomic analysis to establish the chromosome locations of the identified deletions, and in the case of I3-54, to examine whether the clones mapped to chromosome 4B, previously identified as determining the resistance effect of I3-54.

Nullisomic-tetrasomic analysis

Chromosome locations of four clones that identified deletions were mapped using Chinese Spring nullisomic-tetrasomic lines representing each of the wheat chromosomes. Three clones identifying deletions in I3-54 and one in I3-27 were mapped (Table 4.2).

Clone No.	Band Size	Enzyme	Mutant Lines showing deletion	Chromosome location
RDA 23	4.4 kb	EcoRV	I3-54, I3-21	7A
	5.1 kb	EcoRV	I3-48	7D
RDA 9	9.3 kb	DraI	I3-54, I3-21	7A
	2.5 kb	DraI	I3-54	5B/6D
RDA X7	17.7 kb	EcoRv	AJW 4/54, I3-30, I3-31	7A
RDA Y12	4.9 kb	DraI	I3-26, I3-27	2D
	3.5 kb	Dra I	I3-32	1B

Table 4.2: Chromosome locations of four RDA clones, identified using Chinese Spring nullisomic-tetrasomic lines, showing the size of the deleted bands (with the respective enzymes), and the lines containing the deletions.

Using nullisomic-tetrasomic analysis of **RDA 23**, deletions in I3-54 and I3-21, identified by a 4.4 kb band with EcoRV, were mapped to chromosome 7A whilst I3-48 was mapped to 7D, as shown in Figure 4.8.

Nullisomic-tetrasomic analysis of **RDA 9** (Figure 4.9) mapped a deletion identified in I3-54 and I3-21 to 7A. An additional 2.4 kb band deleted only in I3-54 mapped to 5B and 6D. The mapping of a single clone to two separate chromosomes may be explained by the existence of unknown deletions in certain lines. Certain Chinese Spring nullisomic-tetrasomic lines are known to carry deletions (K. Devos, pers. comm.). Nulli 5B-tetra 5D, for instance, has deleted segments on chromosomes 5A and 7A. It is possible that RDA has identified some of these deletions in addition to the induced deletions in the mutants.

RDA X7 was successfully mapped using nulli-tetra analysis. A 17.7 kb band (EcoRV) identifying deletions in AJW 4/54, I3-30 and I3-31 was mapped to 7A. Interestingly, Xpsr Glb3 also identifies deletions in the same lines and maps to 7AS. It is therefore possible that RDA X7 also maps close to this loci. Attempts to map **RDA X10** with the nulli-tetra series proved unsuccessful as the deletion is not identifiable in Chinese Spring. The deleted band present on the Hobbit 'sib' deletion blots is not present in Chinese Spring and has no equivalents. The series therefore can not be used.

RDA Y12 identified deletions in three deletion mutants. The I3-32 deletion has been located to chromosome 1B using nulli-tetra analysis, whilst the deletion in I3-26 and I3-27 has been located to 2D, as shown in figure 4.10.

Fine mapping using the "Opata 85 x Synthetic" population

With certain clones, more precise mapping was attempted using the "Opata 85 x Synthetic" mapping population supplied by Mark Sorrells, Cornell University. The original

Figure 4.8: The use of a Chinese Spring nullisomic-tetrasomic lines to map the RDA derived clone, RDA 23. The deletions in I3-54 and I3-21, identified by the 4.4kb band (marked by the lower arrow), map to chromosome 7A. The the deletion in the 5.1 kb band in mutant I3-48 maps to chromosome 7D (marked by the upper arrow). The autoradiograph of the Southern blot shows EcoRV digested DNAs of Chinese Spring and the nullisomic-tetrasomic group 7 lines, with a λ /HindIII digested size marker .

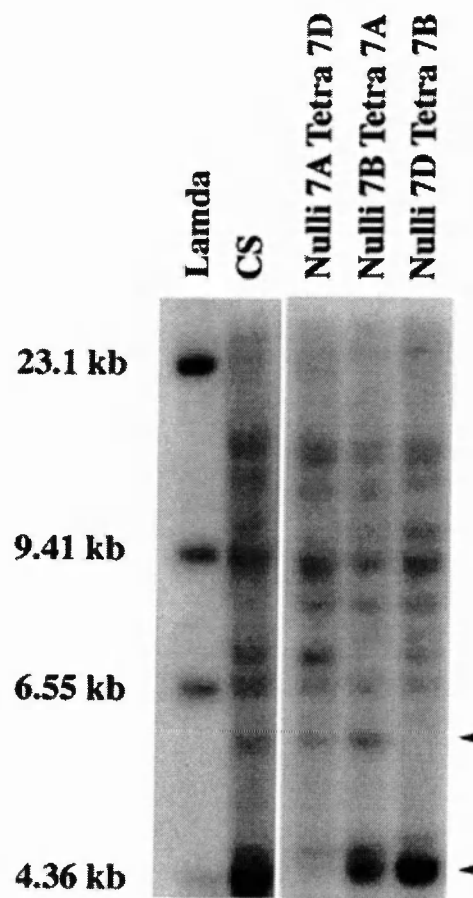


Figure 4.9: The use of a Chinese Spring nullisomic-tetrasomic lines to map the RDA derived clone, RDA 9. The deletions in I3-54 and I3-21, identified by the 9.3 kb band map to chromosome 7A. The deletion in the 2.4 kb band in mutant I3-54 maps to chromosomes 5B and 6D. The autoradiograph of the Southern blot shows DraI digested DNAs of Chinese Spring and the nullisomic-tetrasomic group 5, 6 and 7 lines, with a λ /HindIII digested size marker .

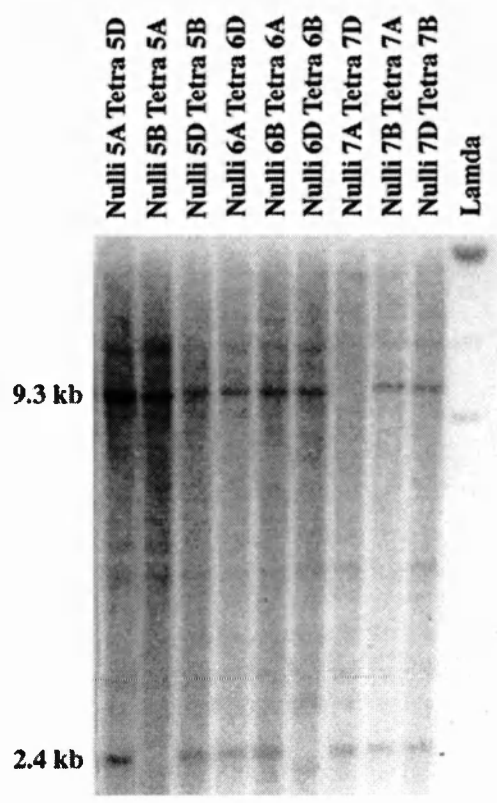
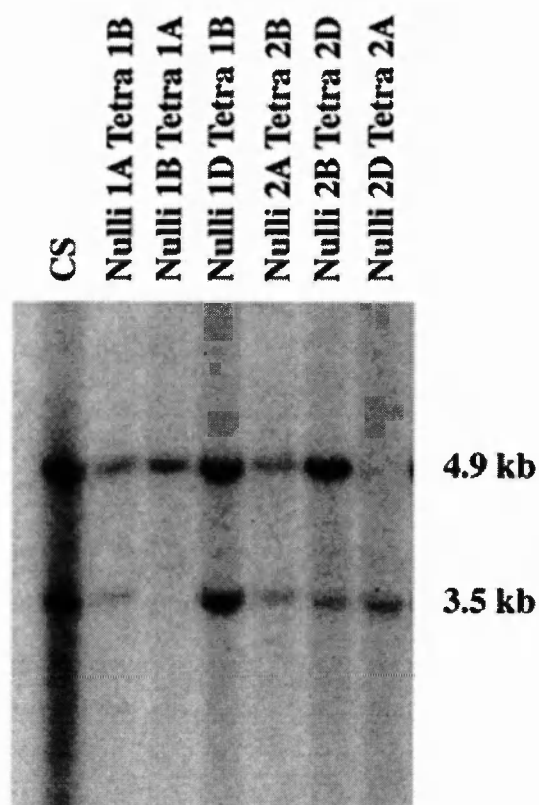


Figure 4.10: The use of a Chinese Spring nullisomic-tetrasomic lines to map the RDA derived clone, RDA Y12. The deletions in I3-26 and I3-27, identified by the 4.9 kb band map to chromosome 2D. The deletion in the 3.5 kb band in mutant I3-32 maps to chromosome 1B. The autoradiograph of the Southern blot shows *Dra*I digested DNAs of Chinese Spring and the nullisomic-tetrasomic group 1 and group 2 lines.



population was set up by crossing a synthetic hexaploid wheat with the spring wheat cultivar 'Opata 85', and the F2 progeny advanced by single seed descent to F7 (Nelson *et al*, 1995). A total of 114 lines were used in the mapping population. In order to identify a suitable enzyme to digest the population with for mapping, multi-enzyme blots using ten enzymes were set up to identify polymorphism between Opata 85 and Synthetic at loci corresponding to the identified deletions. Mapping population filters with the relevant enzyme were then set up and probed with the RDA clone. The mapping lines were scored, depending upon which parental pattern they exhibited. The resulting data was analysed using the programme "Mapmaker". Pairwise comparisons with markers on the chromosome identified by the nulli-tetra analysis were made, and the clones were located to the place of 'best fit' on the chromosome, i.e. the least recombination between adjacent markers.

The multi-enzyme blots were probed with **RDA 9** and polymorphism was identified when using Sst1. The mapping population, digested with Sst1, was probed with RDA 9, and the polymorphisms scored. The deletion found in both I3-54 and I3-21 was found to map to the long arm of chromosome 7A. After pairwise analysis a putative position between mwg380 and ksuD2 is suggested, with recombination values of 11.4% and 9.9% respectively between the two flanking markers and RDA 9 (Figure 4.11).

When RDA 9 is inserted between the two flanking markers, the summation of the recombination frequencies and therefore the genetic distance between them increases over the published distance. However the map detailed by Nelson *et al* (1995) is effectively a compromise of marker similarity and position, and as such is flexible. It is likely that the introduction of an extra marker will cause changes in the previous map. Also, there are quite a number of missing data points in the original data set for the Cornell map so that any marker placement cannot be

Figure 4.11: The use of the 'Opata 85 x Synthetic' mapping population to identify the map position of RDA 9 on chromosome 7AL. The map shows the position of the 'Opata 85 x Synthetic' markers, with RDA 9 (indicated by the arrow) inserted between fba350 and wg380, together with the genetic distance between markers, measured in cM.

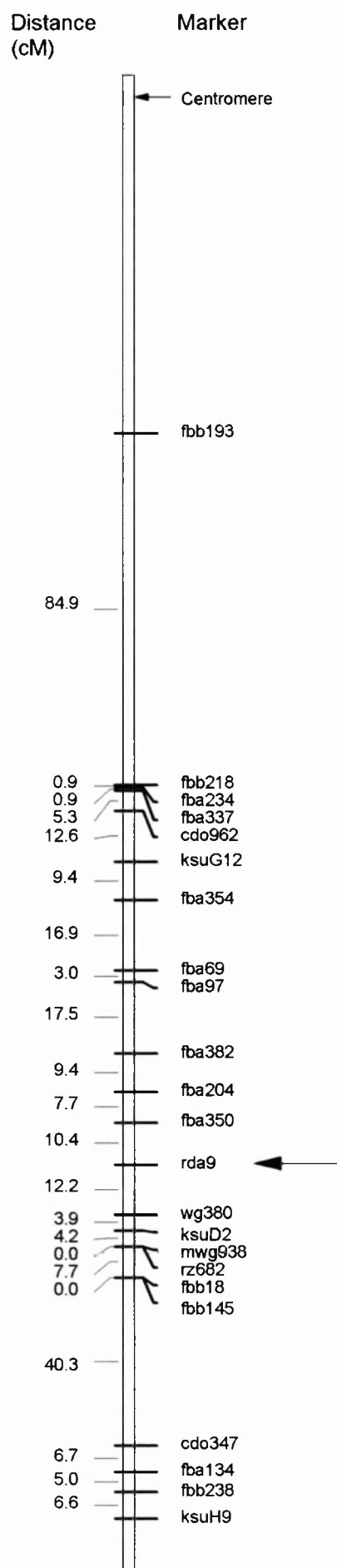
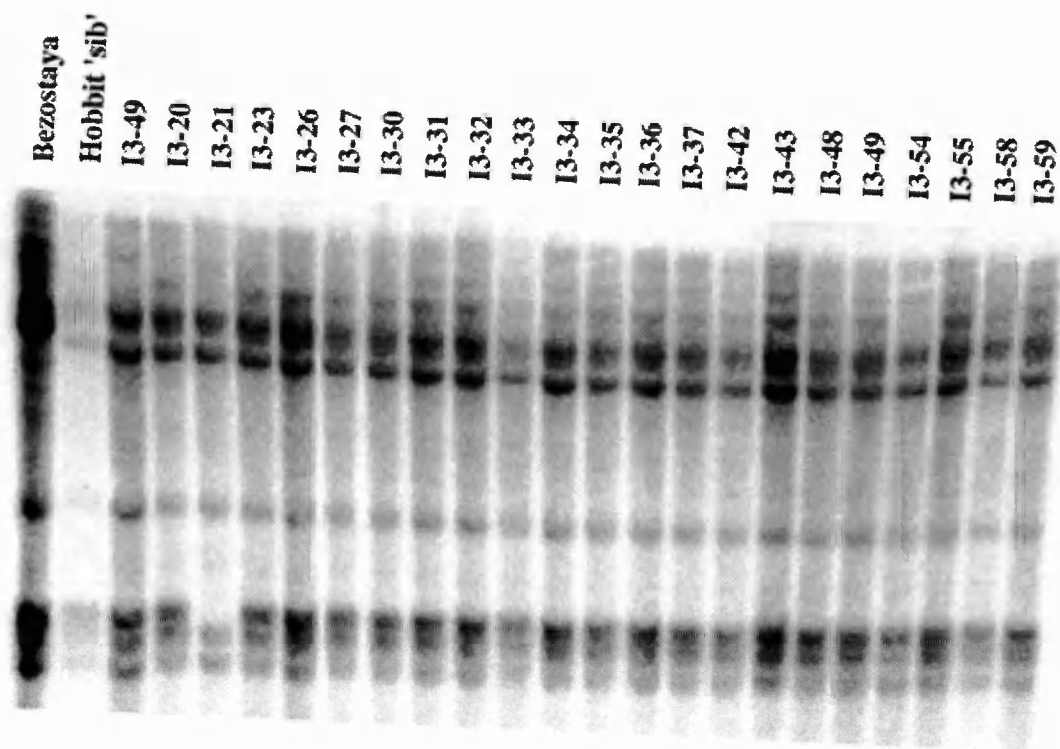


Figure 4.12: The use of the 'Opata 85 x Synthetic' probe Mwg380 (adjacent to RDA 9 on chromosome 7AL) to detect deletions in mutants I3-21 and I3-54 in the bands marked by arrows. The autoradiograph of the Southern blot shows EcoRV digested DNAs of 21 mutant lines with Hobbit 'sib', Bezostaya and Chinese Spring controls.



completely definitive.

Confirmation of the map position of the deletion in I3-54 and I3-21 was obtained by using a marker adjacent to the hypothesized RDA 9 map location on chromosome 7AL. A deletion mutant population was screened with clone "mwg380". As can be seen from figure 4.12, a deletion has been identified in I3-54 and I3-21. If required, further probes in this area of the map could be used to assess the extent of the deletion.

4.5 Mutant Allelism

A number of mutants with increased yellow rust resistance have been identified. It was of interest to establish whether resistance was due to mutations in common genes for susceptibility which would result in allelism of resistance between mutants. Alternatively, the resistance may have been due to independent genes for susceptibility. This was tested by analysing F2 progeny of crosses between selected mutants. If resistance in two mutant parents is due to a mutation in the same gene for susceptibility, there should be no segregation of F2 progeny outside the parental extremes.

Mutant parents with good levels of yellow rust resistance relative to Hobbit 'sib' were selected where either chromosome locations of resistance had been identified through monosomic analysis (section 1.6.3) or map locations of deletions were known. These are detailed in Table 4.3. Where possible, parents were selected where either the resistance location or the deletion location in the two parents were on the same chromosome or chromosome group. It was hypothesized that this may increase the likelihood of the resistance effects in the two mutants being allelic.

F2 progeny from each cross were grown in the field in 1995/96. 10 - 20 plants were sown per 0.6m row, with a Vuka spreader row adjacent to each test row. Plot layout was as described

Cross		Mutant Parents							Susceptible segregants
		Name	Flag leaf infection	Deletion location	Resistance location	Name	Flag leaf infection	Deletion location	
I3-54 x I3-58	I3-54	5%	?	4B	I3-58	10%	?	4BS	14
I3-32 x I3-21	I3-32	15%	5DL	5D	I3-21	10%	5BL (7AL)	?	13
I3-54 x I3-48	I3-54	5%	?	4B	I3-48	8%	4DL	4D	12
I3-58 x I3-21	I3-58	10%	?	4BS	I3-21	10%	5BL (7AL)	?	7
I3-58 x I3-32	I3-58	10%	?	4BS	I3-32	15%	5DL	5D	6

Table 4.3: Results of the mutant allelism tests, showing the crosses tested, the parents and the number of susceptible segregants identified for each cross. The presence of susceptible segregants in each cross indicates that the none of the crosses tested are allelic. Details of the parents are also shown, including the % flag leaf infection of the parental controls in the allelism trial, together with the chromosomal locations of the deletions where identified and the location of resistance effects identified by monosomic analysis of the mutants (Worland & Law, 1991).

in section 2.3.6. Populations were sown in blocks, with control lines adjacent to or within each block. Individual plants were scored for % flag leaf yellow rust infection. All segregants with 50% or more flag leaf infection were classified as susceptible, equivalent to Hobbit 'sib'. The results are given in Table 4.3.

Susceptible segregants were identified in all five test crosses. This demonstrates that none of the five mutant pairs were allelic. This indicates that a number of independent genes for susceptibility may have been mutated or deleted. Analysis of the Hobbit 'sib' monosomic lines identified a number of chromosomes carrying putative genes for susceptibility. In addition, Pink *et al* (1985) suggested that genes for resistance on each of the group 5 short arms of Chinese Spring were balanced by genes for susceptibility on the long arms which, due to their stronger effect, conferred susceptibility to the euploid. Mutation in any of these susceptibility genes may therefore confer resistance. However, it is possible that amongst the 17 mutant lines confirmed to be more resistant than Hobbit 'sib', some of those are allelic, i.e. the resistance is due to the mutation of a single gene for susceptibility. As not all mutant resistance loci have been identified, allelism tests have not been carried out for all possible combinations of these 17 mutants.

Allelism of deletions could be very useful for closer mapping of putative genes for susceptibility. Allelism between two overlapping deletions in separate mutants could be used to identify more precisely the region in which the gene is located. The overlapping segment might be smaller than either of the two original deletions and thus would provide a better target for gene tagging and isolation.

4.6 Discussion

Using a combination of RFLP markers, and markers produced by the RDA technique, locations of deletions for 12 of the 21 mutant lines have been identified (summarised in table 4.4). The majority of deletions have been located on the long arms of the group 4, 5 and 7 chromosomes. However, as the majority of the RFLP marker screening concentrated on these groups, this may suggest that a similar intensity of screening with probes specific to the other chromosome groups may yield similar numbers of deletions.

Several chromosomes were indicated by monosomic analysis of the mutants to have an effect on resistance (Worland & Law, 1991). Deletions in the relevant mutants have been located on several of these chromosomes. Monosomic analysis of I3-32 and I3-48 indicated a resistance response on 5D and 4D respectively. Deletions on the long arms of these chromosomes in the respective mutants have been identified using RFLP markers. Deletions have also been identified on other chromosomes in certain mutants, however, that have not been implicated in the suppression of resistance. A deletion in I3-48 on 7D has been identified using RDA, as well as on 4D, implicated in resistance control. In the case of I3-54, whilst monosomic analysis indicated the resistance effect to be located on 4B, markers on 7A and 6B have been identified, but not on 4B.

Seven of the 12 mutants with identified deletions have more than one deletion, usually on separate chromosomes. It is very likely that each mutant line contains several deletions, potentially scattered over the genome, as in the case of I3-30. It is therefore very important not to assume that all deletions have a role in resistance. The existence of these multiple deletion lines highlights the importance of checking the segregation of the deletions with resistance.

Several deletions were common to a number of mutants, as identified using both RFLP

Table 4.4: Summary of probes identifying deletions in the Hobbit 'sib' mutants. The identity of the RFLP markers and RDA clones shown to be deleted in the mutants are shown, together with their map locations (where known). The map locations (shown in brackets) of certain clones have been predicted, based segregation work detailed in Chapter 5.3.4. The proposed locations of the factor(s) causing resistance in certain mutants, based on monosomic analysis of the mutants (Worland & Law, 1991), are also shown.

Mutant	Resistance location- Monosomic analysis	RFLP marker	Map location	RDA clone	Map location
I3-20		Xpsr 360	5BL	RDA X10	?
I3-21		Xpsr 360	5BL	RDA X10	?
		Xpsr 547	7AL	RDA 9	7AL
				RDA 23	7A
				RDA 4	(7A)
				RDA 22	(7A)
I3-26				RDA Y12	2D
I3-27				RDA Y12	2D
I3-30		Xpsr 115	5BL	RDA X10	?
		Xpsr 370	5BL	RDA X7	7A
		XEmbp	5BL		
		Xpsr 662	6AS		
		XGlb3	7AL		
		Xpsr 563	7AS		
I3-31		Xpsr 662	6AS	RDA X10	?
		XGlb	7AL	RDA X7	7A
		Xpsr 563	7AS		
I3-32	5D	Xpsr 911	5DL	RDA P25	?
		Xpsr 912	5DL	RDA Y12	1B
		Xpsr 574	5DL		
I3-33		Xpsr 360	5BL		
		Xpsr 375	4BL		
		Xpsr 154	4BL		
		XCat	4BL		
I3-48	4D	Xpsr 104	4DL	RDA 23	7D
		Xpsr 163	4DL		
		XFbp	4DL		
I3-54	4B			RDA 23	7A
				RDA 9	7AL
					5B/6D
				RDA 4	(7A)
				RDA 22	(7A)
I3-55		Xpsr 920	4BL		
I3-59				RDA P1	?

and RDA markers. The existence of a common deletion in I3-30 and I3-31 is not particularly surprising as they are sister lines developed from the same original seed. However, there are other cases where common deletions occurred in unrelated lines. It may be possible that certain regions of the genome are more prone to deletions. This may be due to a structural property of the DNA, or it may be that certain areas of the genome are 'weaker' or less stable. Matassi *et al* (1992) proposed that DNA methylation, associated with high GC areas of the genome, acts to stabilise DNA in the chromosome. If this is the case, it may be that the common deletions occur in less methylated, more unstable areas of the genome. Alternatively, if these common deletions confer resistance, it is possible that selection for resistance has resulted in the selection of several lines containing them.

The deletions identified to date using RFLP marker screening have all been found using one source of markers, namely the Xpsr markers from the Cambridge Laboratory (CL) map. However, deletions have not been identified in all mutants. As will be noted from the Figure 4.1 summary, there are certain areas of the genome where markers are lacking. The map tends to have markers clustering around the centromere, with fewer markers at the distal ends of the chromosome. This is particularly noticeable on the group 4 chromosomes, where several potential resistance effects in the deletion mutants have been identified. It is possible that deletions associated with resistance may well be in areas of the genome not covered by the Xpsr markers. It would therefore be useful to use markers from other mapping populations or microsatellites. The map described by Nelson *et al* (1995) would appear to have a number of markers along the long arms of 4BL and 4DL which may serve this purpose. A number of microsatellites have now been developed and mapped onto the CL map (V. Korzun, pers.comm.). Microsatellites being developed at IPK, Gaterslaben, Germany are being mapped onto both the Cambridge Laboratory

map and the "Opata 85 x Synthetic" map (V. Korzun, pers. comm). These would provide a useful additional source of markers. The combination of several sources of markers would ensure better coverage of the chromosomes.

The lack of markers in certain regions of 4B, the proposed location of the I3-54 resistance effect, may be the reason why no deletion in chromosome 4B of this line was not identified. However, even with more markers, the deletion may well prove hard to detect. If the deletion is relatively small, it could be hard to detect with general screening unless closely mapping markers are available.

The existence of several deletions in one mutant line may present problems when using the RDA technique. At least one deletion has been identified in I3-54. If this deletion, or any other that may exist, is larger than the 'target' deletion, it is likely that the proportion of clones derived from the 'target' deletion will be small in relation to the larger deletion clones. Competition in the RDA PCR reactions may also explain the lack of certain clones. It is possible that certain sequences are 'outcompeting' others for the available primers, hence the lack of sequences corresponding to certain potential deletions. The use of the 4B backcross lines should enhance the likelihood of extracting 4B clones, and hence the proposed target sequence. Two backcross drivers have been used but no 4B clone has been successfully isolated so far. If the effect is actually due to a point mutation as a result of irradiation rather than a deletion per se, it will be very difficult to detect using either of the screening methods.

The potential of RDA

Representational Difference Analysis has been shown to be a useful means of locating sequence differences directly. It was repeated several times and located differences between the two genomes being compared, and also other deletion mutant lines. The use of the technique's

predecessor, namely subtractive hybridisation, is well documented in the literature (Chasan, 1992). As well as the numerous examples from the animal kingdom, subtractive hybridisation has proved useful in the cloning of many plant genes in different areas and species, from genetic tumours in tobacco (Fujita *et al*, 1994) to enzymes involved in defence responses in tomato (Pautot *et al*, 1993). The literature concerning the use of RDA has less examples from plant studies but there are numerous examples of the use of the technique in the animal kingdom. As well as numerous examples of mapping of specific sequences, the technique has been used to construct whole genetic maps, such as the rat genetic map constructed by Toyota *et al* (1996). RDA has been used by Delaney *et al* (1995) in a targeted mapping strategy in rye to isolate new RFLP markers for the long arm of chromosome 6. Using this technique, they have isolated markers linked to Hessian fly resistance, known to be present on chromosome 6RL. In their paper on the analysis of fast-neutron generated mutants in *Arabidopsis thaliana*, Bruggemann *et al* (1996) suggest that RDA would provide a useful means of extracting clones for sequences corresponding to deleted genes. The technique has been used to isolate clones specific to deletions proposed to confer resistance of the wheat cultivar 'Guardian' to mildew (Kinane *et al*, 1996), although they have not been screened for co-segregation for resistance.

It is clear that there is potential in the use of RDA for targeting deletions resulting from mutant studies. Although it provides a means of extracting markers for the deletion, further characterisation of the deletion may be more approachable using conventional map markers. A means of achieving this would be to map the RDA clone onto a conventional map and then use markers close to the map location to screen the mutant and thus build up a more comprehensive characterisation of the deletion. The potential of this approach was demonstrated using the mwg380 probe adjacent to RDA 9, as discussed in section 4.4.

Future improvements

The efficiency of RDA in extraction of clones for deletions could be improved. The choice of PstI as the restriction enzyme used to construct the 'representation' of the tester and driver populations was based on two main points. Firstly, PstI is known to be methylation sensitive. It has been shown that methylation plays an important role in the regulation of gene expression during development (Eden & Cedar, 1994). For example, the removal of methylation during vernalization has been proposed to release the block to flowering initiation (Burn *et al*, 1993). In other words, lack of methylation tends to be associated with gene expression. Non-methylated clusters of cytosine residues, known as "CpG islands" have been shown to be associated with genes in higher plant DNA (Antequera & Bird, 1988). Furthermore, work by Moore *et al* (1993a) has shown that in cereal genomes, under-methylated regions of the genome are useful indicators of genes and single copy sequences. Therefore, the use of a methylation sensitive enzyme such as PstI should give a closer representation of the sections of the genome containing genes. Much of the methylated, non-expressed regions of the genome will not be represented in the resulting digested fragments. However, as noted by Lisitsyn *et al* (1993), the use of only one enzyme gives a representation of only part of the genome. Although that is desirable in terms of expressed regions of the genome, the use of alternative methylation sensitive enzymes would provide alternative representations which may provide other deletion specific clones.

In each experiment, only two rounds of RDA were performed on each line, in order to achieve deletion specific clones quickly. However, it will be noted that of the single copy sequences cloned, only a few were deletion specific. Further rounds of RDA would probably increase the proportion of specific sequences in the clones obtained, as Lisitsyn *et al* (1993) have

shown that reiterations of the process have "led to exponentially increasing enrichments of target". In future experiments, it would probably prove useful to carry out at least three rounds of the process before cloning. Although this will take longer, it should reduce the amount of screening necessary to obtain specific clones and thus be effectively more efficient.

The integration of RDA and conventional map markers has demonstrated the usefulness of both techniques in locating deletions. As well as providing a faster means of targeting sequences of interest, this integration may well increase the number of markers available for other applications. With the integration of the suggested improvements, a greater number of deletion specific clones should be attainable. However, as discussed earlier, problems such as competition in the PCR reactions may prevent the detection of certain deletions using RDA. General screening using RFLP markers and microsatellites may enable the detection of these deletions.

CHAPTER 5

SEGREGATION ANALYSIS OF HOBBIT 'SIB' MUTANT RESISTANCE

5.1 Introduction

The work reported in the previous two chapters has dealt with two aspects of the deletion mutants, namely the type and level of disease resistance, and the identification and mapping of the deletions. The initial rationale for generating the deletion mutants was to remove or alter expression of genes for susceptibility identified in the parent variety Hobbit 'sib'. It was the aim of the work described in this chapter to investigate whether any of the induced deletions segregated with resistance, and thus which deletions were responsible for determining resistance. The identification of deletions that segregated with resistance would also corroborate the theory that the resistance exhibited by the mutants was due to the removal of a gene for susceptibility, rather than the induction of a resistance gene.

Monosomic analysis of a number of mutant lines had previously identified the chromosome location of some of the induced changes conferring resistance (Worland *et al*, 1991). The mutants were backcrossed onto Hobbit 'sib' monosomics and the progeny of selfed monosomics were evaluated for yellow rust infection. Analysis of I3-54 indicated that the gene(s) responsible for the resistance effect, and presumably arising from induced mutation, was located on 4B. Evaluation of the Hobbit 'sib' 4B monosomic line had previously identified a promoter of susceptibility on this chromosome (Worland & Law, 1991; Fig: 6.1). Analysis of an F2 cross between Hobbit 'sib and I3-54 segregated to give 3 resistant :1 susceptible, indicating that the mutant carried a single dominant gene (or mutation) for resistance (Worland *et al*, unpublished).

Restricted monosomic analysis of an additional six mutants (I3-27, I3-32, I3-43, I3-48, I3-49 and I3-58) was carried out and analysed as for I3-54. The mutants were backcrossed onto Hobbit 'sib' monosomics 4B, 5BL-7BL, 4D and 5D, all of which had been identified as carrying genes promoting susceptibility. The chromosomes involved in determining resistance were

identified in three mutants, namely 5D in I3-32, 4D in I3-48 and 5D in I3-58. As the full monosomic series was not tested in these mutants, other chromosomes may also carry factors contributing to resistance in the mutants.

Deletion markers detailed in Chapter 4 were identified on chromosomes implicated in mutant disease resistance. Three deletion markers were identified on 4DL of I3-48. Several markers on 5D have been deleted in I3-32 (see Fig. 4.1). Additional deletions were identified on 7D of I3-48 and 2D of I3-32. Deletions were also identified on 7A and 6D of I3-54, although neither of these chromosomes were previously implicated in the resistance response of this mutant. A number of deletions were identified in other mutant lines, where chromosomes responsible for resistance were not implicated.

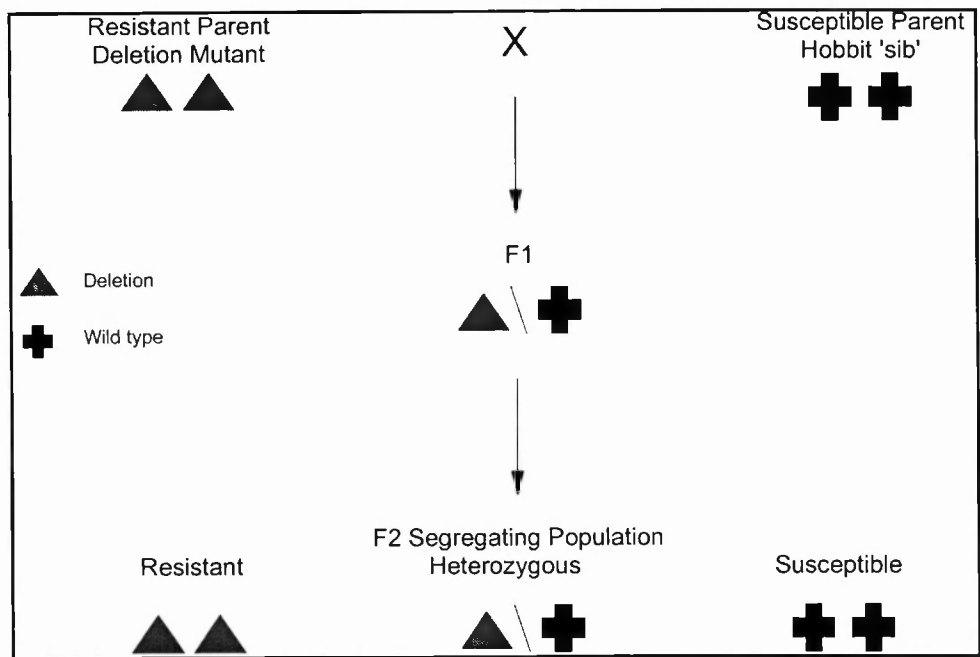


Fig 5.1: Segregation of a linked deletion with resistant segregants in an F2 Hobbit 'sib'/Mutant segregating population.

The role of identified deletions in the mutant resistance response was tested by analysing the segregation of deletion markers identified in Chapter 4 in an F2 segregating population.

5.1.1 Possible Segregation Scenarios

Several basic scenarios exist for the segregation of deletions with resistance.

Single gene segregation

If only one gene for resistance is segregating and the resistance is conferred by the deletion, there will be a significant association between the resistant segregants and the deletion. No susceptible segregants will contain the deletion and no recombinants containing the deletion but lacking the resistance will occur (see Fig: 5.1). Alternatively, if the gene for resistance is independent of the deletion, there will be no significant association and potentially both resistant and susceptible segregants will contain the deletion. However, if the gene is separate from the deletion, but linked to it, there will be a significant association between resistance and the deletion, but susceptible segregants or recombinants containing the deletion may occur, albeit at a low frequency.

Multiple gene segregation

If there are several genes segregating for resistance and none of them are caused by or linked to the deletion, no significant association of resistance with the deletions will occur.

If there is more than one gene segregating for resistance, and one of these is situated in the deletion, or linked to it, identification will depend upon the relative effects of the genes involved. If the gene (or deletion) is essential for resistance and augmented by other genes, a significant association of resistance and the deletion will be demonstrated. However, if the gene has only a partial effect, or must be accompanied by other genes in order to function, a significant association may not be detected, as susceptible segregants containing the gene but lacking the additional genes required for full resistance may occur.

5.2 Materials & Methods

The segregation of deletion specific markers was tested in segregating populations derived from four Hobbit 'sib' mutants, namely I3-27, I3-32, I3-48 and I3-54. Markers specific to 2D in I3-27, 1B and 5D in I3-32, 4D and 7D in I3-48 and 6D and 7A in I3-54 were tested for segregation with the respective mutant resistance.

5.2.1 Segregating Populations

Crosses were made between I3-32, I3-48, I3-54 and Hobbit 'sib'. Analysis of F₂ Hobbit 'sib'/deletion mutant populations involving I3-32, I3-48 and I3-54 was carried out in 1993/94. F₁ crosses were made at the John Innes Centre, Norwich and selfed at PBI Cambridge to give F₂ populations. However, screening with deletion specific markers indicated these populations to be incorrect since none of the deletions known to be present in the mutants could be detected in the respective F₂ populations. It was initially assumed that a field scoring error had occurred but further analysis of these populations indicated an error in the seed stock. These incorrect populations were not analysed further, and the alternative populations involving Rialto rather than Hobbit 'sib', described above, were used for segregation analysis.

Hobbit 'sib' was used as the susceptible cultivar in crosses with I3-27 and the cultivar Rialto was used in crosses with I3-32, I3-48 and I3-54. Although not as susceptible as Hobbit 'sib', Rialto was significantly more susceptible than the mutant lines being tested, and resistant and susceptible segregants could be distinguished from each other. All populations used are summarised in Table 5.1.

Crosses with I3-27

Three different F₂ populations were obtained from the cross of Hobbit 'sib' or equivalent with I3-27, namely JH-A-5, JH-D/27 and JH-16/27. From a total population of 60 plants from

Mutant	Name	Cross	Disease	Year	No. of resistant segregants	No. of susceptible segregants
I3-27	JH-A-5	F2 Hobbit 'sib'/I3-27	YR	93/94	3	3
	JH-D/27	F2 Hobbit 'sib'/I3-27	YR	94/95	6	5
	JH-16/27	F2 Disomic Adw16 (3D)/I3-27	YR	94/95	6	6
I3-32	JH-FR/32	F3 Rialto/I3-32	YR	95/96	13	11
I3-48	JH-R/48	F2 Rialto/I3-48	YR	94/95	6	5
	JH-F3BR/48	F3 Rialto/I3-48	BR	95/96	31	38
I3-54	JH-R/54	F2 Rialto/I3-54	YR	94/95	6	6

Table 5.1: Summary of segregating populations used for the analysis of segregation of deletion markers with resistance to yellow or brown rust, as described in Chapter 5.2.1. The number of resistant and susceptible segregants selected from each population are detailed, together with the year of disease testing of the population and the disease used.

JH-A-5, three resistant and three susceptible segregants were selected. In 1994/95, six resistant and five susceptible segregants were selected from a total of 120 F₂ plants in the JH-D/27 population. An additional six resistant and six susceptible F₂ plants were selected in the JH-16/27 population, again from a total of 120 plants.

Crosses with I3-32

Due to low disease levels in 1994/95, Rialto and I3-32 had very similar disease scores. Therefore, resistant and susceptible segregants could not be readily selected. Twelve plants with low disease scores were however harvested and sown as F₃ ear rows in 1995/96. Two F₃ plants from each row were randomly selected for DNA extraction, and subsequently scored for yellow rust infection at the adult plant stage. These plants make up the JH-FR32 population.

Crosses with I3-48

Despite the low disease levels in 1994/95, I3-48 was readily distinguishable as being more resistant than Rialto and this distinction could be seen in the F₂ segregating population of this cross. Six resistant and five susceptible plants were selected from 120 F₂ Rialto/I3-48 plants (JH-R/48). No leaf tissue was suitable for DNA extraction after disease scoring, so 10 F₃ seeds from each F₂ plant were grown and bulked to represent the F₂ plant genotypes. Seed from the selected lines was grown as single ear rows at F₃ in 1995/96 and scored for yellow rust. Due to low disease levels, this population was not analysed further.

The remaining F₃ seed was used to test for brown rust infection. Ten F₃ plants from each of 4 resistant and 5 susceptible F₂ plants from JH-R/48 were grown under glass house conditions and scored for brown rust infection. This was designated as the JH-F3BR/48 population.

Crosses with I3-54

Six resistant and six susceptible segregants were selected from 120 F₂ plants grown in

1994/95 from the Rialto x I3-54 cross (JH-R/54 population). As with the F2 Rialto/48 population, 10 F3 seedlings from each line were used to represent the individual F2 genotypes.

5.2.2 Disease Testing

Segregating populations were tested for disease resistance by means of either field trials (yellow rust), or conducting tests in glasshouses (brown rust). At the adult plant stage (approximately growth stage 59 to 69; Zadoks *et al* 1974), individual plants were scored for their level of infection and the most susceptible and resistant segregants were selected. Selected segregants were equivalent in disease infection to the susceptible and resistant parents respectively. Leaf material was harvested for later analysis, and grain was harvested once ripe. DNA was extracted from the selected segregant leaf tissue and filters representing the segregating lines plus parental controls were made. These were probed with markers specific for the deletions relating to that population.

Field Tests

For the yellow rust field trials of Hobbit 'sib'/I3-27 F2 populations (varying between 60 and 120 F2 plants), 10 - 20 plants were sown per 0.6 m row, with a Vuka spreader row adjacent to each test row. Plot layout was as described in section 2.3.6. Populations were sown in blocks, with control lines adjacent to or within each block. Control lines were sown to give reference scores for susceptible Hobbit 'sib' and resistant mutant 'types' in the F2 segregating population.

Rialto/Mutant F2 population field trials were sown as a single row of approximately 120 plants per population, again with an adjacent Vuka spreader line.

Glasshouse Testing

One F3 Rialto/I3-48 population was tested under glasshouse conditions (described in

section 2.3.9). At growth stage 47 (late boot stage), plants were inoculated with brown rust. Individual plants were scored at the adult stage and leaf material harvested.

5.2.3 Chi² test for Independence

A null hypothesis of independence of segregation of deletions and resistance was tested by the Chi² test for a 2x2 contingency table (Snedecor & Cochran, 1989), using the Chi² (X²) statistic = $\sum(f-F)^2/F$, where f equals the observed numbers in each cell, and the F s are the expected numbers based upon the null hypothesis being correct. The null hypothesis asserts that the probabilities of resistant and susceptible segregants containing the deletion are equal. In such a case, the resistant and susceptible classes will segregate independently of the deletion. If, on the other hand, the deletion is linked to resistance, a large proportion of the resistant progeny should contain the deletion, whilst a large proportion of the susceptible progeny will lack the deletion. This will result in a significantly high Chi² test indicating that the null hypothesis is incorrect and segregation is not independent of the deletion.

5.3 Results

Poor disease levels in 1994 caused problems when analysing the segregation of relevant deletion markers. Environmental conditions, including high temperatures and low water levels prevented the full development of yellow rust in the 1993/94 trial. Although this made scoring into susceptible and resistant segregants difficult for most crosses, distinct susceptible and resistant segregants were identified in the Hobbit 'sib' x I3-27 population (JH-A-5). Tests were carried out over two further seasons (1994/95 & 1995/96) to test for segregation of deletions markers with resistance in four mutants, namely I3-27, I3-32, I3-48 and I3-54.

5.3.1 Hobbit 'sib' x I3-27

The RDA marker Y12, specific to a deletion on 2D, was used to test all three F2 populations derived from I3-27 (Table 5.2). Average parental scores for 1994 and 1995 differed between 20 and 30 % flag leaf infection for yellow rust (Table 3.2), making resistant and susceptible segregants easily identifiable.

MARKER	POPULATION	GENOTYPE	RESISTANT	SUSCEPTIBLE
RDA Y12	JH-A-5	DELETION	1	1
		WILD TYPE	2	2
	JH-D/27	DELETION	2	4
		WILD TYPE	4	1
	JH-16/27	DELETION	1	0
		WILD TYPE	5	6

Table 5.2: Segregation of RDA Y12 Marker in F2 Hobbit 'sib'/I3-27 Populations

The deletion identified by Y12 was present in both the resistant and susceptible lines (Fig 5.2) selected from the F2 plants of JH-A-5 and JH-D/27. Only one line out of twelve in the JH-16/27 population contained the deletion, and this was classified as resistant. A combined Chi² analysis of the results indicated that the resistance to yellow rust segregated independently of the 'Y12' deletion ($X^2_{[1]} = 0.28$; $P=0.6$).

A Chi² on the combined data from the three F2 populations for differences between rows (deletion vs wild type) indicated that the deletion was transmitted with lower frequency than the wild type 'allele' ($X^2_{[1]} = 4.17$; $P=0.05 - 0.01$).

5.3.2 Rialto x I3-32

Thirteen resistant segregants and eleven susceptible segregants were selected from the F3 Rialto/I3-32 population. Eight of the resistant segregants were derived from four homozygous

Figure 5.2: Use of RDA derived clone, RDA Y12, to investigate the segregation of the 2D deletion in I3-27 in a F2 Hobbit 'sib'/ I3-27 segregating population. The deletion was identified in 2 resistant segregants and 4 susceptible segregants, indicating it is not linked to resistance in I3-27. The autoradiograph of the Southern blot shows EcoRV digested DNAs of Rialto, I3-27 plus the resistant and susceptible F2 plants selected from the JH-D/27 population, with a λ /HindIII digested size marker .



resistant F3 families and six of the susceptible segregants were derived from three homozygous susceptible F3 families. The remaining five segregants in each group were derived from five segregating and therefore heterozygous F3 families. However, no controls were tested, so that true resistant and susceptible extremes equivalent to the parents could not be determined. Therefore, the results of this analysis must be taken with some caution. The F3 population may also be biased in that the original F2 plants were selected as being resistant and were not a random selection. The true susceptible extremes may therefore not be present in this population.

The JH FR/32 segregants were probed with two markers specific to deletions on 5DL, Xpsr 911 and 574. These two markers are relatively closely linked on 5DL and it is likely that they are part of the same deletion in I3-32. The RDA clone Y12, marking a deletion on 1B in I3-32, was also tested (Table 5.3).

MARKER	POPULATION	GENOTYPE	RESISTANT	SUSCEPTIBLE
XPSR 911 & XPSR 574	JH-FR/32	DELETION	2	2
		WILD TYPE	11	9
RDA Y12	JH-FR/32	DELETION	4	4
		WILD TYPE	9	7

Table 5.3: Segregation of I3-32 markers in JH-FR/32 population

Two resistant segregants, with 5% flag leaf infection, and two susceptible segregants, with 50 - 80% infection lacked the Xpsr 911 marker, suggesting this marker was not linked with resistance. This was confirmed by X² analysis (X²_[1]=0.035;P=0.8). A similar result was obtained when using the Xpsr 574 marker, also located on 5DL. The identical segregation pattern exhibited by both markers supports the claim that both markers are linked and part of the same deletion.

When tested with RDA Y12, four resistant and four susceptible segregants contained the

deletion. These results also indicate that Y12 is not linked to the resistance and segregates independently, again confirmed by a low X^2 value of 0.084 ($P=0.7$).

The row totals for all three markers indicate that the 5DL and 1B deletions were transmitted with lower frequency than the wild type genotype, although only the 5DL deletion is significant ($X^2_{[1]}=10.69;P=0.01 - 0.001$).

5.3.3 Rialto x I3-48

Yellow rust tests

The F2 JH-R/48 population, in which each F2 plant was represented by a bulk of 10 F3 seedlings, was tested with three markers (Table 5.4). Only one resistant segregant contained the 7D deletion identified by RDA 23, indicating this marker is not linked to resistance ($X^2_{[1]}=0.917;P=0.3$). The row X^2 indicates that the 7D deletion was transmitted at lower frequency than the wild type in the F2 progeny ($X^2_{[1]}=7.36; P=0.01 - 0.001$).

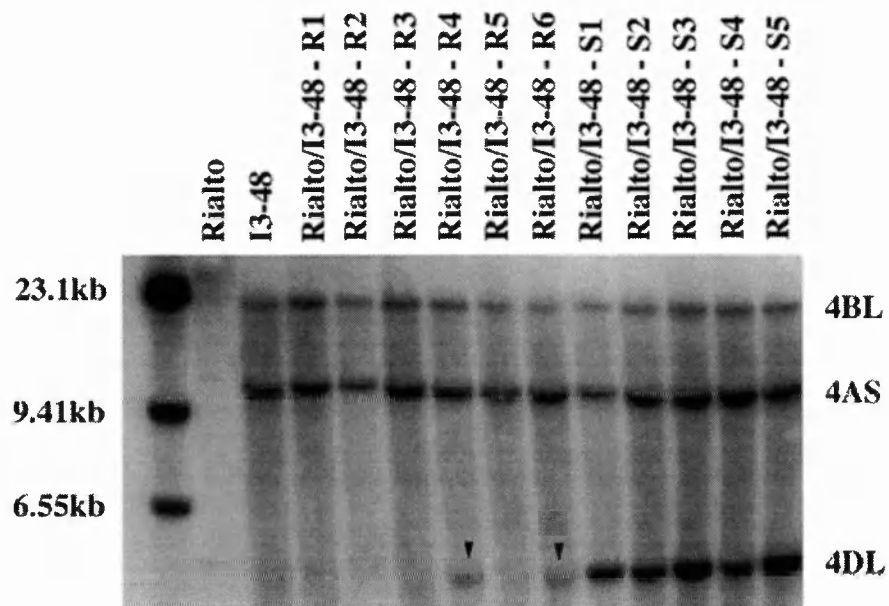
Xpsr 104 and 163, identifying a deletion on 4DL, both segregated with resistance, as deletions were identified in all 6 resistant segregants but not in any of the susceptible lines, suggesting the markers are linked to resistance. This is confirmed by the high X^2 value ($X^2=11.04; P=0.01$).

MARKER	POPULATION	GENOTYPE	RESISTANT	SUSCEPTIBLE
XPSR 104 & XPSR 163	JH-R/48	DELETION	6	0
		WILD TYPE	0	5
RDA 23	JH-R/48	DELETION	1	0
		WILD TYPE	5	5

Table 5.4: Segregation of I3-48 Markers in the JH-R/48 Population

However, at longer autoradiograph exposures, faint bands corresponding to 4DL could be

Figure 5.3: Use of RFLP Xpsr 163 to investigate the segregation of the 4DL deletion in I3-48 in an F3 bulked Rialto/ I3-48 segregating population. The deletion was detected in all 6 resistant segregants but no susceptible segregants, suggesting the deletion is linked to the resistance. However, faint bands can be detected in Rialto/I3-48-R4 and Rialto/I3-48-R6, indicated by arrows, suggesting not all plants in those two bulks contain the deletion. The autoradiograph of the Southern blot shows DraI digested DNAs of Rialto, I3-48 plus the resistant and susceptible F3 bulks of Rialto/I3-48 F2 progeny, with a λ /HindIII digested size marker.



detected in two of the resistant F2 lines (Fig 5.3), indicating that one or more of the F3 plants bulked to represent the F2 plants contained the wild type allele. Ten individual F3 plants from each of these two lines were analysed with Xpsr 163. Two F3 plants in each of the two bulks contained the wild type allele. There are two possible explanations for this. First, the plants may be outcrosses with adjacent Hobbit 'sib' type plants or the spreader Vuka. Whilst an outcross to a Hobbit 'sib' type would be difficult to detect, outcrossing with Vuka was checked using the polymorphic marker Xpsr 934. None of the F3 progeny showed the Vuka polymorphism, suggesting no outcrossing with Vuka had occurred. Secondly, the two F2 lines in question may have been heterozygous for the deletion, and thus segregated non-deleted F3 plants. Analysis of additional F3 plants, derived from the JH-F3BR/48 population, from one of the two resistant families under investigation showed that 6 out of 20 of the F3 plants analysed contained the wild type allele. This frequency is too high to be attributable to outcrossing, suggesting the F2 plants were heterozygous when selected.

An F3 population containing all the resistant and susceptible families derived from the F2 plants was tested for yellow rust resistance in the field in 1995/96. However, due to poor establishment of yellow rust, the susceptible parent, Rialto, was indistinguishable from the resistant I3-48 parent. Therefore differences in the families were not detected and further testing of the segregation of yellow rust resistance with the deletion could not be carried out.

The segregation of the deletion, marked by Xpsr 163 and 104, with resistance is consistent with the deletion being causal for resistance to yellow rust. This result must be further corroborated by segregation analysis at F3. This will enable the confirmation of heterozygosity in the two F2 resistant lines, R4 and R6, as some susceptible plants lacking the 4DL deletion will segregate in the F3 progeny of those lines. F3 analysis should also give further evidence of the

4DL deletion being causal.

Brown rust tests

A glasshouse test was carried out with the remaining F3 seed (JH-F3BR/48). Families derived from four yellow rust resistant and five yellow rust susceptible F2 plants were tested. The segregation of two of the 4DL deletion markers (Xpsr 104 and 163) was determined. Results of the analysis are summarised in Table 5.5 and detailed in Table 5.6. The majority of the F3 resistant lines, selected as resistant to yellow rust at F2, contained the deletion and were resistant to brown rust, whilst most of the susceptible lines lacked the deletion, and were susceptible to brown rust, suggesting the deletion on 4DL is linked to brown rust resistance ($X^2_{[1]}=61.4$; $P<0.001$).

MARKER	POPULATION	GENOTYPE	RESISTANT	SUSCEPTIBLE
XPSR 104 & XPSR 163	JH-F3BR/48	DELETION	31	2
		WILD TYPE	0	36

Table 5.5: Segregation of I3-48 4DL Markers in a F3 Rialto/I3-48 Segregating for Brown Rust Resistance

A number of brown rust susceptible lines containing the deletion were identified in one family identified as susceptible to yellow rust at F2. The family is designated S3 in Table 5.6. Cytological examination of the family indicated that a number of telosomic and monosomic plants were found in at least three of the lines that had previously been identified as containing the deletion. This suggests that the F2 plant was probably cytologically aberrant, and that the identified 'deletions' may well be a product of this aberrance. This family was therefore removed from further analysis and has not been included in Table 5.5.

However, two further brown rust susceptible F3 segregants containing the deletion were

Table 5.6: Detail of segregation of the 4DL deletion in I3-48 with brown rust resistance in a F3 Rialto/I3-48 segregating population. The specific F3 plant code, the brown rust phenotype and the presence or absence of the deletion are detailed. The line code indicates the identity of the F2 plant from which the F3 plant was derived, e.g. S64 is the fourth plant in the F3 family derived from the susceptible F2 plant, S6.

Line	Phenotype	Deletion	Line	Phenotype	Deletion
R21	R	D	S11	S	W
R22	R	D	S12	S	W
R23	R	D	S13	S	W
R24	R	D	S14	S	W
R25	R	D	S15	S	W
R26	R	D	S16	S	W
R27	S	D	S17	S	W
R28	R	D	S18	S	W
R29	R	D	S19	S	W
R210	R	D			
R31	R	D	S31	S	?
R32	R	D	S32	S	W
R33	R	D	S33	S	D
R34	R	D	S34	S	W
R35	R	D	S35	S	W
R36	R	D	S36	S	D
R37	R	D	S37	S	W
R38	R	D	S38	S	W
R39	R	D	S39	S	W
R310	R	D	S310	S	D
R41	R	D	S41	S	W
R42	R	D	S42	S	W
R43	S	W	S43	S	W
R44	S	W	S44	S	W
R45	S	W	S45	S	W
R46	R	D	S46	S	W
R47	S	W	S47	S	W
R48	R	D	S48	S	W
R49	R	D	S49	S	W
R410	R	D	S410	S	W
R51	R	D	S51	S	D
R52	R	D	S52	S	W
R53	R	D	S53	S	W
R54	R	D	S54	S	W
R55	R	D	S55	S	W
R56	R	D	S56	S	W
R57	R	D	S57	S	W
R58	R	D	S58	S	W
R59	R	D	S59	S	W
R510	R	D	S510	S	W
			S61	S	W
			S62	S	W
			S63	S	W
			S64	S	W
			S65	S	W
			S66	S	W
			S67	S	W
			S69	S	W
			S610	S	W

R=Resistant
S=Susceptible

D=Deletion
W=Wild Type

also identified; one originated from an F2 line resistant to yellow rust (R2), the other from an F2 line susceptible to yellow rust (S5). Cytological examination of both these families indicated they were both normal. Whilst the segregant originating from the susceptible family, designated S5-1, had a disease score (25% flag leaf infection) only slightly more than the I3-48 parental confidence interval, the susceptibility of the segregant derived from the resistant family, designated R2-7, was significantly greater than any of the resistant lines. Analysis of F4 progeny of R2-7 confirmed the high disease level of the line and the presence of the deletion. In addition, F4 progeny of an additional F4 family derived from the same F2 resistant plant (R2) segregated, with 50% of the plants showing susceptibility to brown rust. All the lines contained the deletion. Other F4 lines from the same original F2 plant, such as R2-5 and R2-9 remained resistant.

The existence of these recombinant susceptible lines at F3 and F4 suggests that the deletion identified by Xpsr 104 and 163 may not be causal but is linked to resistance to brown rust, as confirmed by the significant χ^2 analysis.

5.3.4 Rialto x I3-54

The JH-R/54 population was probed with five deletion specific RDA markers. All five markers identified deletions in the same two susceptible segregants, suggesting that all five markers are linked (Table 5.7). This would suggest that all five markers are in close proximity to the RDA 9 locus reported in section 4.4. In addition to the deletions identified by the 7A band of RDA 9, deletions were identified in one resistant segregant and three susceptible segregants on the 6D (or 5B) band. The segregation of this band confirms the presence of two separate and genetically distinct deletions in I3-54. The results show that neither deletions segregate with resistance. This is confirmed by the non-significant χ^2 values of 2.40($P=0.2 - 0.1$) and 1.5 ($P=0.2$).

MARKER	POPULATION	GENOTYPE	RESISTANT	SUSCEPTIBLE
RDA 23, 22, 18, 4 & RDA 9 (7A)	JH-R/54	DELETION	0	2
		WILD TYPE	6	4
RDA 9 (6D)	JH-R/54	DELETION	1	3
		WILD TYPE	5	3

Table 5.7: Segregation of I3-54 Markers in a F2 Rialto/I3-54 Population Segregating for Yellow Rust Resistance

5.4 Discussion

The complete segregation at F2 of the 4DL deletion in I3-48, marked by Xpsr 104 and 163, with yellow rust resistance indicates the deletion may be causal for yellow rust resistance. If so, this suggests that the deletion has inactivated a gene for susceptibility, conferring resistance to the mutant. Therefore, Xpsr 104 and 163 could effectively be markers for a gene for susceptibility in Hobbit 'sib'. However, in light of the results of the F3 segregation of the deletion with brown rust resistance, further analysis of F3 segregation of the 4DL deletion with yellow rust resistance is required before it can be concluded beyond any doubt that the 4DL deletion is causal for yellow rust resistance.

The presence of the F3 plant, S5-1, that is homozygous for the 4DL deletion but is susceptible to brown rust also has implications for the role of the deletion in yellow rust resistance. For the deletion to be homozygous at F3, it must have been present in the heterozygous form in the yellow rust susceptible F2 S5 plant. If the 4DL deletion is causal for yellow rust, this indicates that the deletion may be recessive and is only fully effective in the homozygous state. Alternatively, the disease level may be due to a deletion dosage effect, as has previously been noted in a comparison of euploids, monosomics and nullisomics, where decreasing dosage of chromosomes conferring susceptibility results in progressively increased

resistance (Pink *et al*, 1983). Therefore, an F2 plant heterozygous for the deletion may have a resistance level intermediate between the homozygous resistant and homozygous susceptible phenotypes. The S5 plant may therefore represent a heterozygous F2 plant that has been mistakenly selected as a fully susceptible plant. If this is the case, F3 analysis of this plant for both yellow rust and brown rust resistance should indicate resistance to yellow rust but susceptibility to brown rust. This would demonstrate that the 4DL deletion is causal for yellow rust resistance but only linked to brown rust resistance. However, if the F3 segregant was susceptible to both yellow rust and brown rust, this would indicate that the deletion is linked to resistance to both diseases, but not causal for either.

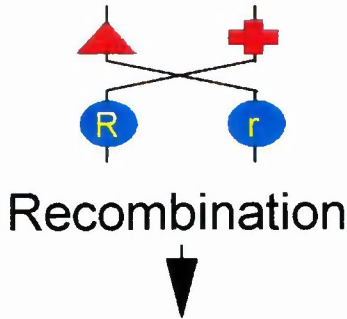
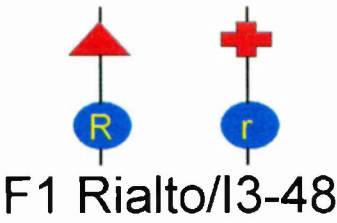
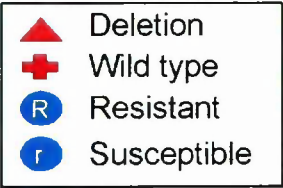
5.4.1 Recombination

The presence of two F3 plants that are susceptible to brown rust but contain the deletion indicate that the 4DL deletion is not causal for brown rust, although linkage of the deletion with brown rust resistance has been demonstrated. The two F3 recombinants, R2-7 and S5-1, can be explained as the result of a cross-over event in the F1. The two recombinants represent different scenarios in terms of segregation of deletions and resistance. R2-7 has lost the resistant factor but maintained the deletion, whilst S5-1 has presumably never possessed the resistance factor but has gained the deletion. Both recombinants can be explained by an appropriate cross-over taking place in the F1. This is illustrated in Fig 5.4. It assumes that the deletion is separate but linked to the brown rust resistance factor, and that this is dominant. Recombination between the two loci at F1 breaks the linkage, and can lead to independent segregation of the resistance and the deletion in certain F2 plants.

Recombinant line R2-7 -

An F2 recombinant plant that is homozygous for the deletion, but heterozygous for the

Figure 5.4: Model of a potential recombination event at F1 in a Rialto/I3-48 cross, resulting in segregation of F3 segregants that are brown rust susceptible but homozygous for the deletion, as described in Chapter 5.4.

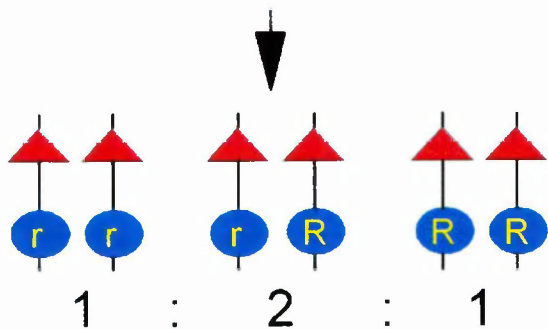
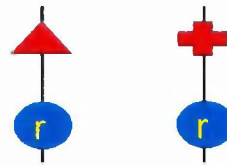
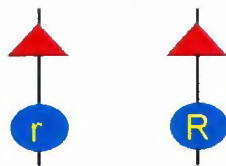


F1 Gametes



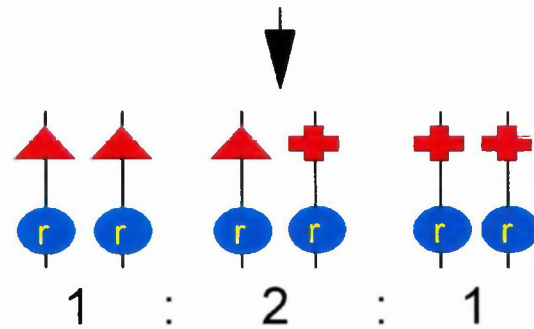
F2 Rialto/I3-48
Resistant

F2 Rialto/I3-48
Susceptible



R-2-7

F3



S-5-1

resistance factor will be selected as resistant. Segregation in the F3 will therefore give a 1 susceptible : 2 heterozygous : 1 resistant ratio (assuming normal segregation) for the resistance factor, with a homozygous deletion. The majority of the plants will therefore be resistant, but some susceptible segregants containing the deletion will segregate, such as R2-7. This model is further corroborated by the presence of susceptible F4 segregants in the R2-8 line. This result would suggest the R2-8 was heterozygous for resistance at F3, as R2-7 was at F2, and segregates for resistance in F4. Several homozygous resistant lines have also been identified at F4, including R2-5 and R2-9.

Recombinant line S5-1 -

An F2 recombinant plant that is homozygous recessive for resistance, but heterozygous for the deletion will be selected as susceptible. At F2, the presence of the wild type allele will hinder the detection of the deletion. Segregation in the F3 will give a 1 deletion: 2 heterozygous : 1 wild type segregation for the deletion, but homozygous recessive for the resistance factor. Therefore, the majority of the plants will be susceptible and contain the wild type allele, but some segregants will have the deletion, such as S5-1.

5.4.2 The use of segregation analysis

The presence of recombinants in the F3 highlights the usefulness of testing segregating populations at F3 as well as F2 in situations where resistance segregates with the deletion. Recombinants would not have been clearly identified at F2 in the test of the I3-48 deletion. F3 analysis of brown rust resistance has allowed the identification of recombinants, demonstrating that the segregation is due to linkage.

It should be noted that the F3 analysis was carried out with brown rust, not yellow rust. It is possible that recombinants only occur with brown rust resistance. If the resistance of the

deletion mutant to several diseases is actually due to a cluster of mutations, each specific for a different disease, it is possible that the marked deletion is actually causal for yellow rust resistance but only linked to brown rust resistance. F3 segregation with yellow rust was not available due to poor establishment of the disease in the field trial.

Ideally, a second, independent segregating population should be tested for resistance to yellow rust and brown rust. It would be preferable to use a Hobbit 'sib' x I3-48 population as the background would be more homogeneous, and would increase confidence in the observed effects being attributable to the deletion, or lack of it. The use of Hobbit 'sib' as a susceptible control would provide a truly susceptible line, rather than the intermediate susceptibility of Rialto. This would make easier the classification into susceptible and resistant segregants. In addition, segregation analysis of deletions would be more reliable in F2 populations derived from Hobbit 'sib', as there is less likelihood that background susceptibility genes, introduced by a non-Hobbit 'sib' parent, will be segregating and confounding the effects of the deletion.

In conclusion, the results clearly demonstrate linkage of the 4DL deletion in I3-48 to resistance to yellow rust and brown rust. Whilst the F2 analysis indicates the deletion is causal for yellow rust resistance, this conclusion can not be unequivocal until an F3 analysis of the segregation of the deletion with yellow rust resistance has been carried out. If susceptible segregants homozygous for the deletion are detected at F3, the deletion is not causal. However, if no such segregants are identified, it can be concluded that the deletion is causal. The results of the F3 analysis for segregation with brown rust resistance demonstrate that the deletion is not causal for brown rust resistance, but is linked to it.

5.4.3 Agreement with resistance location via monosomic analysis

Monosomic analysis of the resistance of certain deletion mutants had previously identified

chromosome locations for the resistance factors (Worland & Law, 1991). The comparison of monosomic analysis and deletion analysis is summarised in Table 5.8.

There is agreement between the monosomic analysis and deletion location for I3-48. In I3-48, resistance was mapped to 4D. The 4DL deletion segregates with resistance, suggesting the two are linked. Monosomic analysis did not indicate 7A to be involved in the resistance of I3-48. The deletion on 7A does not segregate with the resistance.

There was strong evidence for resistance of I3-54 to be located on 4B (loc.cit) but no deletion was found on 4B for segregation analysis. As expected from the monosomic analysis, neither of the deletions on 7D or 6D segregated with resistance.

Mutant	Location of resistance (Monosomic Analysis)	Deletion Location (RFLP or RDA analysis)	Linkage of deletion with resistance
I3-32	5D	5DL	No
		1B	No
I3-48	4D	4DL	Yes
		7A	No
I3-54	4B	7D	No
		6D (5B)	No

Table 5.8: Comparison of Monosomic Analysis and Deletion Segregation Results

The resistance of I3-32 was mapped to 5D. Therefore, the lack of segregation of the 1B marker with resistance was expected. However, initial results indicated the 5DL deletion did not segregate with resistance either. Although not a thorough test, as explained in section 5.3.2, initial work suggested the marker was not linked. It is therefore possible that the deletion causing the 5D resistance of I3-32 is located elsewhere on the chromosome. Alternatively, it is possible that Rialto lacks the 5DL gene for susceptibility found in Hobbit 'sib' and deleted in I3-32. If this is the case, the segregation ratios would not fit the expected frequencies for a linked marker, as the

deletion would not be conferring resistance to the Rialto parent.

Monosomic analysis of I3-27 has not identified a chromosome location for the resistance factor. Therefore, a comparison of the resistance and deletion locations can not be made.

5.4.4 Lower frequency transmission of deletions

It was noted that in all cases where deletions were not linked to resistance, the frequency of transmission was lower than that of the wild type allele. It is possible that the deletions affect the pairing ability of the chromosomes or interfere in some way with meiotic segregation such that the deletions are transmitted at a lower frequency. Alternatively, the deletions may actually be deleterious and affect pollen viability or embryo vigour. In each of the crosses examined in this study, the mutant has always been used as the male parent. Transmission of the deletion may be affected by which parent it is derived from, and may be favoured by one parent rather than the other. This situation has been noted before in studies of transmission of a major 5D deletion in wheat (Atkinson *et al*, 1995). Male and female transmission studies of plants heterozygous for the deletion chromosome indicated 9.5% and 48.8% transmission through the pollen and the egg respectively. Poor transmission of deletions through the male parent has also been noted in *Arabidopsis thaliana* (Bruggemann *et al*, 1996). Using the mutant as the female parent may therefore alleviate the problems of biased transmission.

5.4.5 Future work

As previously noted, F3 analysis of the segregation of the 4DL deletion in I3-48 with yellow rust resistance is necessary to determine whether this deletion is causal. The identification of markers more closely linked to brown rust resistance would also be useful. The recombinant lines may provide source material for bulk segregant analysis using the RDA procedure to isolate markers specific to brown rust resistance. The recombined segregants offer the potential to trace

the actual source of resistance as, in a bulk, they are genetically very similar, including the linked deletion, but will differ for the resistance allele between susceptible and resistant segregants. RDA analysis of the bulks from the same F2 resistant family, such as F3 bulks of R2-5 and the susceptible recombinant R2-7, may enable the isolation of clones specific and linked to brown rust resistance.

5.4.6 Conclusions

The use of segregation analysis has proved a useful tool in identifying deletions associated with disease resistance in deletion mutants. The crucial factor in this analysis is the presence of susceptible lines containing the deletion. It is theoretically possible to misdiagnose plants without the deletion as resistant as a result of disease escape. Only susceptible segregants containing the deletion will disprove the causal effect of the deletion. The identification of a number of deletions in the mutant populations that are not associated with resistance highlights the necessity to check all deletions with the relevant segregating population in order to identify linked deletions. Deletions not segregating with resistance have no usefulness in terms of disease response and may even be deleterious. Markers may therefore be of use for two purposes in breeding programmes. They could enable the tagging of deletions conferring resistance, and facilitate the removal of extraneous deletions. For example, the marker RDA 23 could be used as a tag to remove the 7A deletion from I3-48.

The aim of the work was to investigate and, if possible, demonstrate co-segregation of deletions with resistance. Co-segregation of the 4DL deletion with yellow rust resistance, and linkage to brown rust resistance has been demonstrated. The 4DL deletion markers, Xpsr 104 and Xpsr 163, should therefore provide a useful tag for the mutation induced resistance in I3-48, and would enable the selection of lines containing this character in a breeding population.

CHAPTER 6

DISTRIBUTION OF GENES FOR SUSCEPTIBILITY IN VARIETIES OTHER THAN HOBBIT 'SIB'

6.1 Introduction

Analysis of the monosomic series in Hobbit 'sib' was used to identify chromosomes that played a role in the variety's adult plant resistance to yellow rust (Worland & Law, 1991). Twelve chromosomes were identified that significantly altered the level of infection when in the monosomic state, as illustrated in Fig. 6.1. Seven monosomic lines had a significantly elevated level of disease relative to the euploid control, indicating the presence of genes for resistance on those chromosomes. Five monosomic lines had significantly reduced levels of infection relative to the Hobbit 'sib' control. This indicated that there were genes for susceptibility on chromosomes 3B, 4B, 4D, 5BL-7BL and 5D of Hobbit 'sib'. The reduced dosage of these genes in the monosomic lines gave a reduced level of infection.

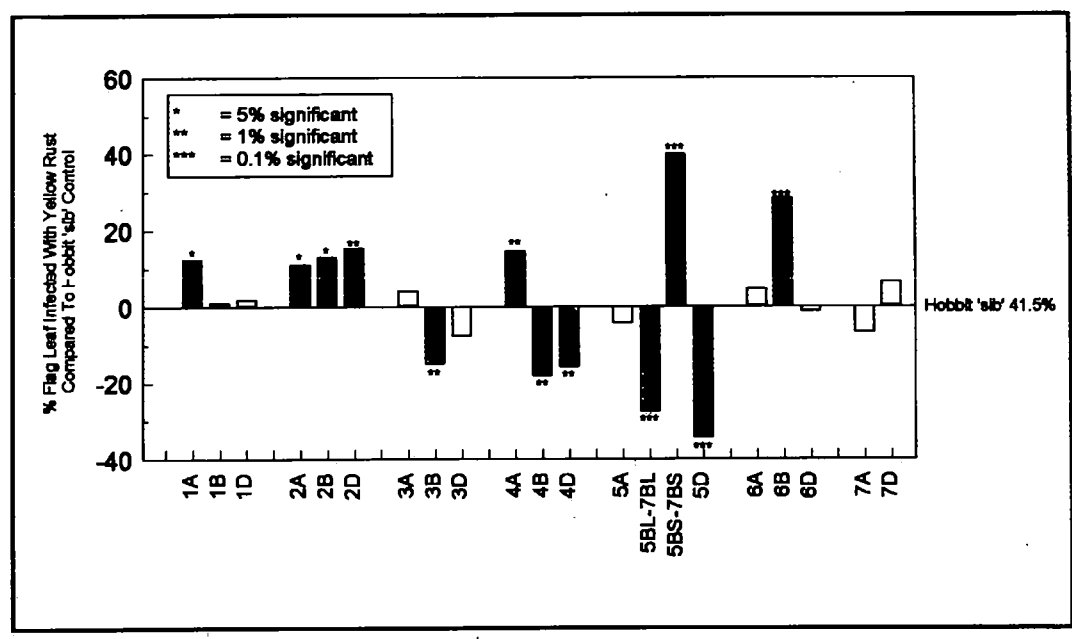


Fig 6.1: Percentage yellow rust infection on flag leaves of Hobbit 'sib' monosomic lines, relative to Hobbit 'sib'

In an attempt to inactivate the genes for susceptibility, irradiation of Hobbit 'sib' was carried out using fast neutrons. A number of mutants were derived with increased resistance to

yellow rust relative to the Hobbit 'sib' control, as detailed in chapter 3. The improved resistance of these mutants may suggest that genes promoting susceptibility have been inactivated.

The purpose of the work detailed in this chapter was to establish whether genes for susceptibility are present in other cultivars. This was tested by comparing the effects of specific cultivar chromosomes with homologous chromosomes previously reported to increase susceptibility in Hobbit 'sib'. Two methods were used to achieve this.

First, the presence of genes for susceptibility in the cv. Bezostaya were investigated by disease testing the F2 progeny of crosses between Hobbit 'sib'/Bezostaya substitution lines and certain deletion mutants thought to contain inactivated genes for susceptibility. This tested for allelism between specific Bezostaya chromosomes substituted into Hobbit 'sib', and homologous chromosomes in the deletion mutants. Secondly, the distribution of genes for susceptibility in seven UK commercial varieties was analysed using a backcross reciprocal monosomic analysis, which theoretically allowed the comparison of the effects of specific varietal chromosomes with their Hobbit 'sib' homologues previously shown to carry genes for susceptibility.

6.1.1 Bezostaya substitution line allelism

Investigation of Hobbit 'sib'/Bezostaya substitution lines (A.J. Worland, 1991; Johnson, 1992) demonstrated the improvement of resistance of Hobbit 'sib' by the substitution of certain Bezostaya chromosomes, as illustrated in Fig 6.2.

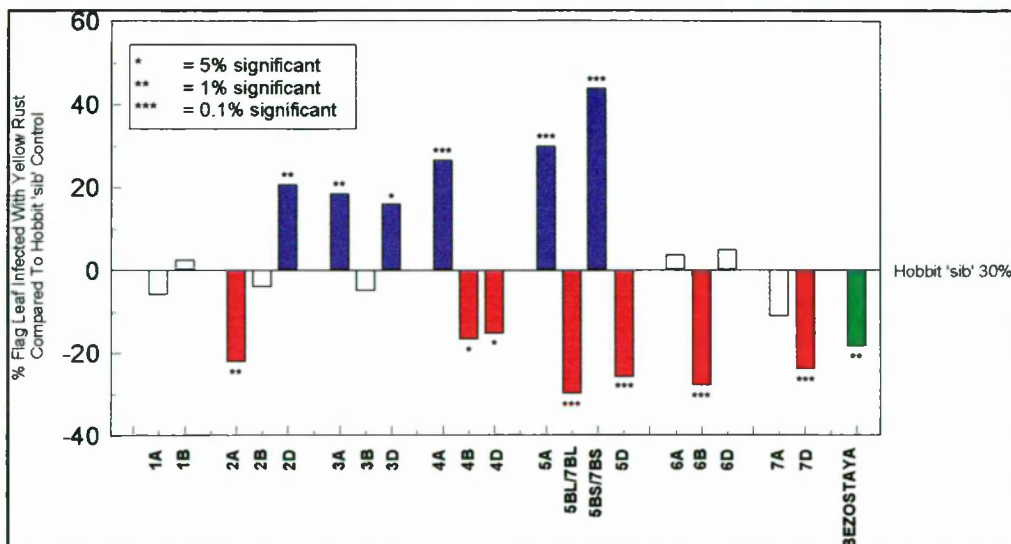


Fig 6.2: Percentage yellow rust infection on flag leaves of Hobbit 'sib'/ Bezostaya substitution lines, relative to Hobbit 'sib'

The introduction of the Bezostaya chromosomes 2A, 4B, 4D, 5BL-7BL, 5D, 6D and 7D significantly improved the resistance of the recipient variety. Chromosomes 4B, 4D, 5BL-7BL and 5D had all previously been shown to carry genes for susceptibility in Hobbit 'sib'. It was therefore proposed that the improvement in resistance in the substitution lines could be due to the introduction of weaker susceptibility genes or a lack of genes for susceptibility from Bezostaya, at homologous loci to the Hobbit 'sib' alleles. Alternatively, the increased resistance may have been due to the addition of Bezostaya resistance factors.

The deletion mutants are also presumed to be more resistant due to the loss of Hobbit 'sib' susceptibility genes. If improved resistance in both the substitution lines and the deletion mutants was due to loss of Hobbit 'sib' genes for susceptibility, it would therefore be interesting to investigate whether the Bezostaya alleles were allelic, to the deletion mutant resistance. This was tested by examining the segregation of F2 populations of Bezostaya substitution x deletion mutant crosses. The demonstration of allelism between mutant and Bezostaya alleles would be

useful in several ways. First, it would give additional evidence supporting the theory that Bezostaya carries weaker alleles for susceptibility. Secondly and more practically, the deletions would provide markers that could be used to follow the Bezostaya alleles through a breeding programme. The Bezostaya alleles may also provide closer markers for genes for susceptibility than is possible using deletion markers. Whilst the gene for susceptibility may be located in the deletion, if the deletion is large, markers identifying the deletion may actually be distant from the susceptibility locus. The Bezostaya allele may be a more precise marker within the region covered by the deletion.

6.1.2 Backcross Reciprocal Analysis of Commercial Varieties

Genes for susceptibility have been demonstrated in a number of older cultivars. Work with the monosomics of Chinese Spring, Bersee and Cappelle-Desprez has demonstrated genes for susceptibility on a number of chromosomes (Pink *et al*, 1983; Kema *et al*, 1995; Law *et al*, 1978). Whilst the work on these various monosomic series has demonstrated some correlation in terms of chromosome location for the promoters of susceptibility and resistance in the different varieties, it is also clear that there is variation between varieties. As detailed above, there are 6 chromosomes in Bezostaya and Hobbit 'sib', namely 4A, 4B, 4D, 5BL-7BL and 5D which have a similar effect on the resistance response of both varieties. Other chromosomes, such as 7D, have an effect in Bezostaya, but not in Hobbit 'sib'.

Very little is known of the distribution of genes for susceptibility in modern commercial cultivars. It is possible that modern breeding techniques may have caused the loss of susceptibility genes through the selection of lines with increased background resistance. Alternatively, alleles for reduced susceptibility may have an adverse effect on certain agronomic characters and thus lead to the selection against the more 'resistant' alleles. The demonstration of

genes for susceptibility in modern cultivars, and the subsequent tagging and removal of those genes from future varieties would represent significant progress in terms of breeding for resistance, as it may reduce the reliance on major genes for resistance to disease.

One possible means of investigating the distribution of genes for susceptibility would be to develop monosomic series or substitution lines in the variety of interest. However, the long development times involved to obtain these stocks are rather prohibitive in terms of a relatively short term project. The time factor also restricts the number of varieties that can be tested. The identification of Hobbit 'sib' monosomic lines carrying genes for susceptibility offers the opportunity to compare commercial varietal chromosomes with these lines to identify homologous alleles. A technique first described by Snape & Law (1980), known as backcross reciprocal monosomic analysis, was therefore used as a faster means of directly comparing chromosomes of interest from commercial varieties with their homologues in Hobbit 'sib'. The method involves using monosomic lines for the chromosomes of interest in a two generation crossing programme, generating reciprocal lines with different homologous chromosomes but similar backcross backgrounds. Any variation between the two backcross reciprocal families could therefore be attributed to the chromosomes of interest, and thus identify similarities or differences between the varietal and Hobbit 'sib' chromosomes. The technique is summarised in Figure 2.2.

6.2 Bezostaya/Hobbit 'sib' Mutant allelism

Previous work with Hobbit 'sib'/ Bezostaya substitution lines had shown that certain substitution lines showed a reduction in the level of yellow rust infection relative to Hobbit 'sib', suggesting the introduction of resistance alleles from Bezostaya, such as *Lr34 /Yr18* on

chromosome 7D, or loss of susceptibility alleles present in Hobbit 'sib' (Johnson, 1992). The Hobbit 'sib' mutants had shown an increase in resistance relative to the parental control. Crosses between certain substitution lines and mutants were made and F2 segregating populations were tested to check for allelism between the Bezostaya and mutant 'alleles'. Parental Hobbit/Bezostaya substitution lines 4B, 4D, 5BL-7BL and 5D, previously demonstrated as having improved disease resistance over Hobbit 'sib' (Worland & Law, 1991) were selected for testing for allelism with specific mutants.

Selection of mutant parents was based on two factors. One category of cross was made where monosomic analysis of the mutant had previously identified a resistance effect on the chromosome homologous to the substituted Bezostaya chromosome (Worland & Law, 1991). A second category of cross involved resistant mutant parents where deletions had been located on chromosomes homologous to the substituted Bezostaya chromosome, but whether or not the deletion(s) were responsible for the resistance was unknown. All crosses are summarised in Table 6.1.

A null hypothesis of allelism was set up as follows. For the Bezostaya allele and the mutant 'allele' to be allelic, no F2 or F3 progeny should segregate outside the parental extremes. However, it is recognised that a low percentage of natural aneuploids could occur and segregate outside the parental extremes. This possible source of misclassification will need to be guarded against.

Cross	Hobbit 'sib'/Bezostaya Parent	Mutant Parent	
	Location of Resistance	Location of Resistance	Location of Deletion(s)
Bdw (Bez 4B) x I3-54	4B	4B	7AL
I3-58 x Adw (Bez 4B)	4B	4B	Unknown
Bdw (Bez 4D) x I3-48	4D	4D	4DL, 7DL
Adw (Bez 5D) x I3-32	5D	5D	5DL, 1B
Bdw (Bez 5D) x I3-32	5D	5D	5DL, 1B
I3-21 x Adw (Bez 5BL-7BL)	5BL-7BL	Unknown	5BL-7BL
I3-33 x Adw (Bez 5BL-7BL)	5BL-7BL	Unknown	5BL-7BL, 4BL
I3-33 x Bdw (Bez 4B)	4B	Unknown	5BL-7BL, 4BL

Table 6.1: Location of resistance or deletions in parents used for allelism testing.

6.2.2 Disease testing

Populations of approximately 120 F2 plants were sown for field trialling. For certain crosses, all F2 seed was used in the sowing of trials for 1993/94. Random F3 bulks were harvested from these lines for testing in 1994/95 and 1995/96. 10 - 20 plants were sown per 0.6m row, with a Vuka spreader row adjacent to each test row. Plot layouts were as described in section 2.3.6. Populations were sown in two to three adjacent plots, with one row of each control sown at either end of the population block. Individual plants in each population were scored for % flag leaf yellow rust infection. A mean of each control row was calculated visually.

The number of segregants significantly more susceptible or resistant than the parental extremes was recorded. Parental extremes were calculated as a 95% confidence interval around the control means. The confidence interval was calculated by multiplying the 5% t-value, detailed in Fisher & Yates (1967), by the control population standard deviation. Hobbit 'sib' was used as

the standard susceptible control. In cases of non-allelism between the deletion mutant resistance and the Bezostaya 'resistance' allele, recombination should give plants lacking both sources of resistance and thus should be as susceptible as Hobbit 'sib'.

6.2.3 Results

Difficulties were encountered with the disease scoring of the Hobbit 'sib'/Bezostaya x Mutant segregating populations in 1993-94 due to unusually hot and dry conditions. The environmental conditions meant that levels of infections were low and good sporulations on the leaf were present for only a short time. Therefore, reliable disease scores were only available for the 1994/95 and 1995/96 seasons.

Of the eight segregating families grown for scoring, two families involved crosses with a substitution line that was itself showing severe symptoms of hybrid necrosis. The two families were I3-33 x Adw (Bez 5BL-7BL) and I3-21 x Adw (Bez 5BL-7BL). The segregation of hybrid necrosis in a majority of the F2 segregating population made accurate scoring very difficult so the two lines were removed from further analysis. Analysis was also discontinued for the cross Bdw (Bez 4D) x I3-48 as the substitution line in these experiments, in contrast to previous data, showed similar levels of infection to the susceptible control Hobbit 'sib'. Isozyme testing showed the substituted chromosome to be incorrect (A.J. Worland, pers. comm). Segregants more susceptible than the parental extremes could therefore not be identified. Also the cross of Bdw (Bez 5D) x I3-32 was discontinued as independent tests to verify the identity of the substitution line showed the line to be incorrect.

Four segregating populations remained for detailed analysis, where both the mutant and Bezostaya substitution line control means were significantly more resistant than Hobbit 'sib'. The results are given in Table 6.2.

Line	Chromosome	Year	No. of Plants	Parental %Flag Leaf Infection		No. of Segregants	
				Mutant	Substitution line	Susceptible	Resistant
Bdw (Bezostaya 4B) x I3-54	4B	1995	140	2.5	36.6	7	0
		1996	120	14.06	37.14	9	0
I3-33 x Bdw (Bezostaya 4B)	4B	1995	100	30	36.6	0	0
		1996	61	28.3	37.14	1	0
Adw (Bezostaya 5D) x I3-32 (F3)	5D	1995	90	20	10	9	0
		1996	146	20.71	24.29	5	38
I3-58 x Bdw (Bezostaya 4B)	4B	1995	150	24	37	0	0
		1996	121	18.75	37.14	0	0

Table 6.2: Results of the Hobbit 'sib'/Bezostaya x mutant allelism tests, showing the number of segregants with disease infections outside the 95% parental confidence intervals. Hobbit 'sib' was used as the susceptible control and had a flag leaf infection score of 65%.

Bdw (Bez 4B) x I3-54

A number of segregants were scored in 1994/95 and 1995/96 in the Bdw (Bez 4B) x I3-54 population that were significantly more susceptible than the most susceptible parent. Five percent or more of the population in each year were significantly more susceptible. No significantly more resistant segregants were identified, due to the very low disease level of the mutant parental control.

I3-33 x Adw (Bez 4B)

The highly necrotic nature of I3-33 (Fig. 6.3) made accurate scoring of the F2 progeny in the I3-33 x Adw 4/Bezostaya population difficult, as well as the parental control. As a consequence, the 95% confidence interval of the mutant parent was very large, making it difficult to distinguish statistically more susceptible segregants. One segregant was more susceptible in 1996.



Fig 6.3: Necrotic Hobbit 'sib' mutant I3-33, shown in front of 'Vuka' spreader plants

A number of segregants significantly more susceptible than the susceptible parent were identified in both years of disease testing. However, the population tested was an unbagged, F3 bulk. As such, outcrossing may have occurred in the F2 that became fixed in the F3 and gave rise to the susceptible segregants. As illustrated in Fig 6.4, other than the susceptible segregants, the majority of the population clustered below the substitution line confidence limit. However, there were also a large number of segregants significantly more resistant than the deletion mutant parental control. Including susceptible and resistant segregants, nearly a third of the F3 population was outside the parental extremes.

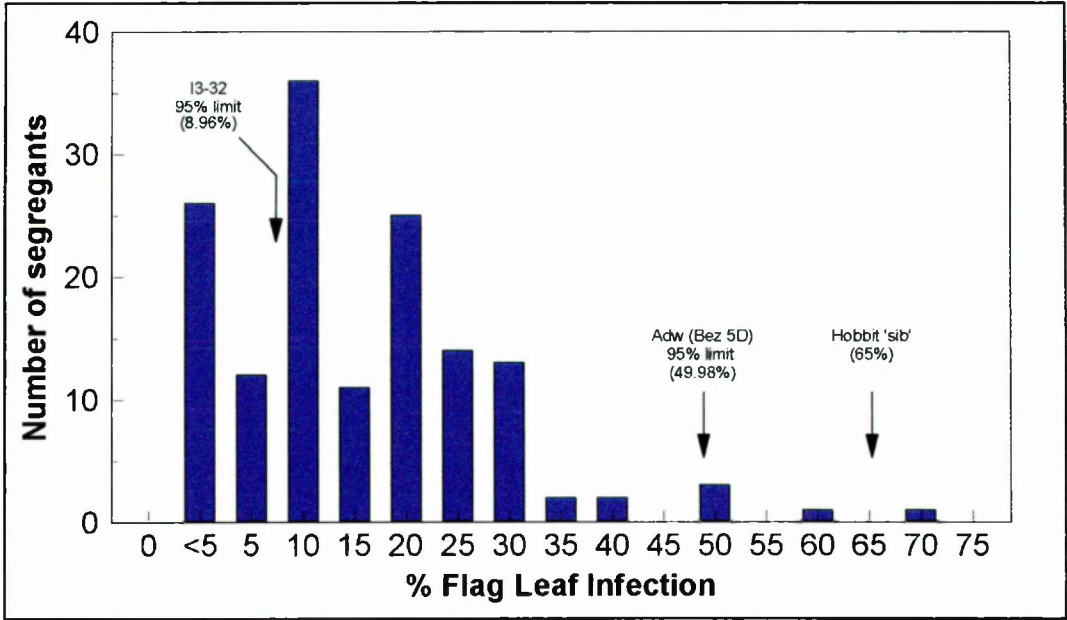


Fig 6.4: Distribution of yellow rust infections in segregants of a 1996 F3 Adw (Bez 5D) x I3-32 Population, showing the upper 95% confidence limit of the Adw (Bez 5D) parent and the lower 95% confidence limit of the I3-32 parent.

I3-58 x BdW (Bez 4B)

No susceptible or resistant segregants outside the parental extremes were found in the I3-58 x BdW (Bez 4B) segregating population. As illustrated in Fig. 6.5, the distribution of segregants for the 1996 population is clustered within the limits of the parental means, with the majority of the population giving infection levels at least 12% below the substitution line confidence limit of 62.35% flag leaf infection. Although the 95% confidence interval limit of the BdW (Bez 4B) substitution line was close to the disease level of Hobbit 'sib' and thus may have made the identification of susceptible segregants difficult, the results from the BdW (Bez 4B) x I3-54 population show that susceptible segregants can be distinguished from the BdW (Bez 4B) parent.

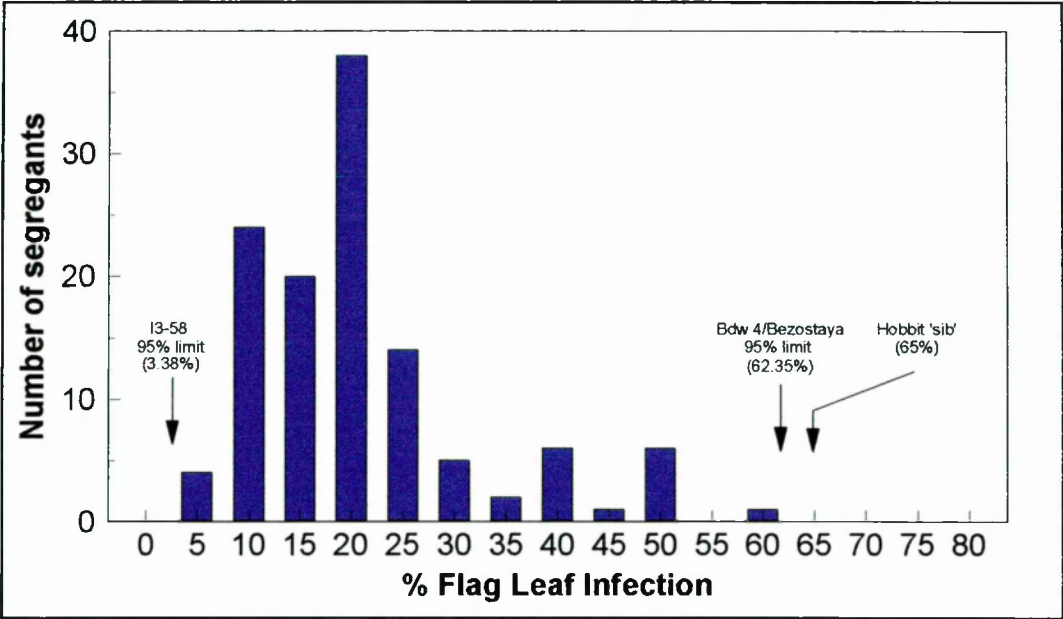


Fig 6.5: Distribution of yellow rust infection in segregants of a 1996 F2 I3-58 x BdW (Bez 4B) Population, showing the upper 95% confidence limit of the BdW (Bez 4B) parent and the lower 95% confidence limit of the I3-58 parent.

6.2.4 Discussion

Due to problems with the parental lines of four of the eight populations originally tested, only four populations were fully analysed.

Problems with parental lines

The unexpectedly high level of disease on the BdW (Bez 4D) parental substitution line prevented the identification of segregants in the BdW (Bez 4D) x I3-48 F2 population. This is unfortunate, as the demonstration of allelism in this population could have exploited the resistance-linked I3-48 deletion markers discussed in Chapter 5 to establish linkage with the Bezostaya allele. Bezostaya chromosome 4D was identified as carrying an allele conferring resistance in an analysis of the Hobbit 'sib'/Bezostaya substitution lines (Worland & Law, 1991). However, the disease level was much lower in the original test, Hobbit 'sib' having a flag leaf infection level of 30%, compared to 65% in the current test. Approximately a 15% reduction in disease level was attributable to Bezostaya 4D in the original test. It is possible that this resistance effect could be due to a gene(s) that was not effective at higher disease pressures.

High levels of necrosis made the Adw (Bez 5BL-7BL) parent difficult to score. It is thought that the necrosis is due to the combination of the necrotic allele Ne1 on chromosome 5BL-7BL of Bezostaya with Ne2 found either in Hobbit 'sib', or possibly on the background 2B chromosome derived from Cappelle-Desprez, since the Hobbit 'sib' monosomics and substitution lines were developed from the Cappelle-Desprez monosomic series. It is therefore possible that the Ne2 allele has come through in the background, causing hybrid necrosis in the substitution line.

Testing of the BdW (Bez 5D) substitution line with markers for grain hardness, isozymes and β -amylase all indicated the parental line was incorrect. Therefore, the population derived

from it was unreliable.

The efficiency of allelism analysis

The use of allelism tests to identify useful alleles in Bezostaya has proved very difficult as problems were experienced with a number of parental lines. Good estimates of parental control means are necessary to thoroughly test the allelism null hypothesis. Large parental confidence intervals will tend to mask transgressive segregation thus leading to the false acceptance of allelism. Therefore, large parental control numbers giving rise to smaller confidence intervals are needed if susceptible segregants and thus non-allelism are to be accurately demonstrated.

The technique also relies on the correct identification of chromosomes conferring resistance in the mutant parent. Full monosomic analysis of chromosomes conferring resistance in the mutants was only carried out for I3-54. For all other mutants studied in the current analysis, only limited monosomic analysis was carried out, investigating a subset of chromosomes. Therefore, the chromosome conferring resistance to these mutants may be one of those not originally analysed.

If allelism can be demonstrated and markers are available for the causal deletion in the deletion mutant, this technique may provide an accurate way of incorporating alleles conferring resistance from Bezostaya into other varieties.

Allelism tests

The presence of segregants outside the parental extremes for two populations, BdW (Bez 4B) x I3-54 and Adw (Bez 5D) x I3-32, indicates that they are not allelic. The identification of 38 significantly resistant segregants in the Adw (Bez 5D) x I3-32 population, in addition to the susceptible segregants, suggests the resistance of the two parents may be additive. Monosomic analysis of I3-54 and I3-32 clearly demonstrated resistance effects on 4B and 5D respectively.

Lack of allelism shows that the resistance effects caused by these two chromosomes in Bezostaya must be at different loci to the deletions. It is also possible that the resistance of Bezostaya in these two cases is due to resistance genes on the investigated chromosomes.

Whilst the results of the I3-33 x Bdw (Bez 4B) population may suggest the deletion and the Bezostaya are allelic, the effect of necrosis on scoring accuracy makes the determination of susceptible segregants difficult. No firm conclusions can be drawn from this population.

Of the four crosses analysed, the results are consistent with the demonstration of allelism in the I3-58 x Bdw (Bez 4B) population only. No segregants with disease levels outside the 95% parental confidence interval were noted. The 95% confidence interval of the Bezostaya substitution line was relatively large, which may have made identification of segregants more susceptible than the control difficult. However, susceptible segregants were identified in the Bdw (Bez 4B) x I3-54 population, which had the same substitution line control, indicating susceptible segregants should be identifiable. Larger parental control populations would enable better estimation of parental means, and thus smaller confidence intervals. This would confirm and give greater confidence in accepting the null hypothesis of allelism between the I3-58 and Bdw (Bez 4B) alleles.

Monosomic analysis of I3-58 has identified a resistance effect on chromosome 4BS, although no deletion has been found using molecular markers. Allelism between the mutant and Bezostaya alleles confirms the previous identification of the 4B resistance effect in I3-58. Identification of the causal I3-58 deletion would provide a marker for the Bezostaya allele, which may allow introduction of the Bezostaya allele, and thus the resistance conferred by it, into a breeding population. Conversely, polymorphism at the Bezostaya allele may also provide a marker that could be used to identify the deletion in I3-58.

6.3 Backcross reciprocal monosomic analysis

Seven commercial varieties of interest to PBI Cambridge breeders were selected for comparison. The varieties exhibited varying levels of disease resistance. Lines monosomic for five chromosomes (3B, 4B, 4D, 5BL-7BL and 5D), identified as carrying genes promoting susceptibility in Hobbit 'sib' were used as tester lines. The aim of the experiment was to carry out backcross reciprocal monosomic analysis for each of the 5 chromosomes in each of the 7 varieties.

The initial crosses involved crossing the tester monosomic, in this case Hobbit 'sib', with the varieties of interest. In the first generation, the tester monosomic parent was used as the female parent. In the progeny of the cross of a variety onto a monosomic test line, all 41 chromosome monosomic progeny will carry the hemizygous unpaired chromosome derived from the male variety parent under test (Sears, 1954). The 20 pairs of background chromosomes of the F1 hybrid will be derived from both parents, one chromosome from each. Monosomic segregants are identified cytologically. In the second round of crossing, F1 hybrid monosomics are crossed both as male and female parents to the monosomic tester line. When the F1 hybrid is used as the male parent, the resulting monosomic progeny will be, almost exclusively, hemizygous for the varietal chromosome, with background segregation for the remaining twenty chromosomes. When the hybrid is used as the female parent, the resulting monosomic progeny will have a similar heterozygous background but the hemizygous chromosome will derive from the tester monosomic, Hobbit 'sib'. The technique is summarised in Figure 2.2.

Two sets of monosomic progeny are therefore produced for each chromosome being tested. These differ only in their hemizygous chromosomes; the backgrounds on average being the same. If these lines are grown in randomised, replicated experiments any differences between

the means of the two sets should be attributable to the differences between the two chromosomes of interest, provided the experiment contains enough sib lines. Where dosage in the monosomic may affect results, the lines can be taken to the next generation so that disomics, homozygous for the alternative homologous chromosomes can be extracted to evaluate the effect of the chromosome.

A working null hypothesis of no significant difference between varietal and Hobbit 'sib' chromosomes was used. This was tested by comparing reciprocal varietal and Hobbit 'sib' family means, and identifying significant differences between them using the formulae detailed in section 6.3.5.

6.3.2 Materials & Methods

Five chromosomes postulated to carry genes for susceptibility, namely 3B, 4B, 4D, 5BL-7BL and 5D, in Hobbit 'sib' were compared with their homologues in the seven varieties.

Varieties

- Genesis: A hard endosperm variety with quality suitable for some bread-making processes. Good resistance to brown rust although somewhat susceptible to mildew and yellow rust.
- Haven: A hard endosperm feed variety. Susceptible to yellow rust and brown rust, although resistant to other foliar diseases.
- Hereward: A hard endosperm variety very suitable for bread-making. Susceptible to yellow rust.
- Hornet: A hard endosperm feed variety. Susceptible to yellow rust and *Septoria tritici*.
- Hunter: A soft endosperm feed variety. Good resistance to yellow rust, brown rust and *Septoria tritici*.

- Rialto: A hard endosperm variety suitable for some bread-making processes. Susceptible to yellow rust and very susceptible to brown rust.
- Riband: A soft endosperm feed variety. Susceptible to yellow rust, brown rust and *Septoria tritici*.

Yellow Rust Races

A three isolate mixture of races was used with virulence against a number of known major resistances in the varieties used, as described in Table 6.3.

Race	Virulence	Varieties affected
41E136(4)	<i>Yr1, Yr2, Yr3, Yr14</i>	Hobbit 'sib'
169E168	<i>Yr1, Yr2, Yr3, Yr9, Carstens V</i>	Rialto, Genesis, Hereward
237E141	<i>Yr1, Yr2, Yr3, Yr4, Yr6, Yr9,</i> <i>(Yr13, Yr14)</i>	Haven, Hornet, Rialto, Riband

Table 6.3: Yellow rust race isolates used, indicating the isolate virulence and the varieties attacked. () indicates possible additional virulence of the isolates.

6.3.3 Development of Reciprocal Monosomic Lines

The aim was to develop backcross reciprocal families for 35 reciprocal comparisons, made up of comparisons between the 5 chromosomes in each of the 7 varieties described in section 5.2 with their Hobbit 'sib' homologues. The development of the lines in this study is summarised in Fig 6.6. Progeny of crosses between the Hobbit 'sib' monosomics and euploid varieties were supplied by A.J.Worland (John Innes Centre). The seven varieties were crossed as the male parent onto Hobbit 'sib' monosomics 3B, 4B, 4D, 5BL-7BL and 5D. Monosomic

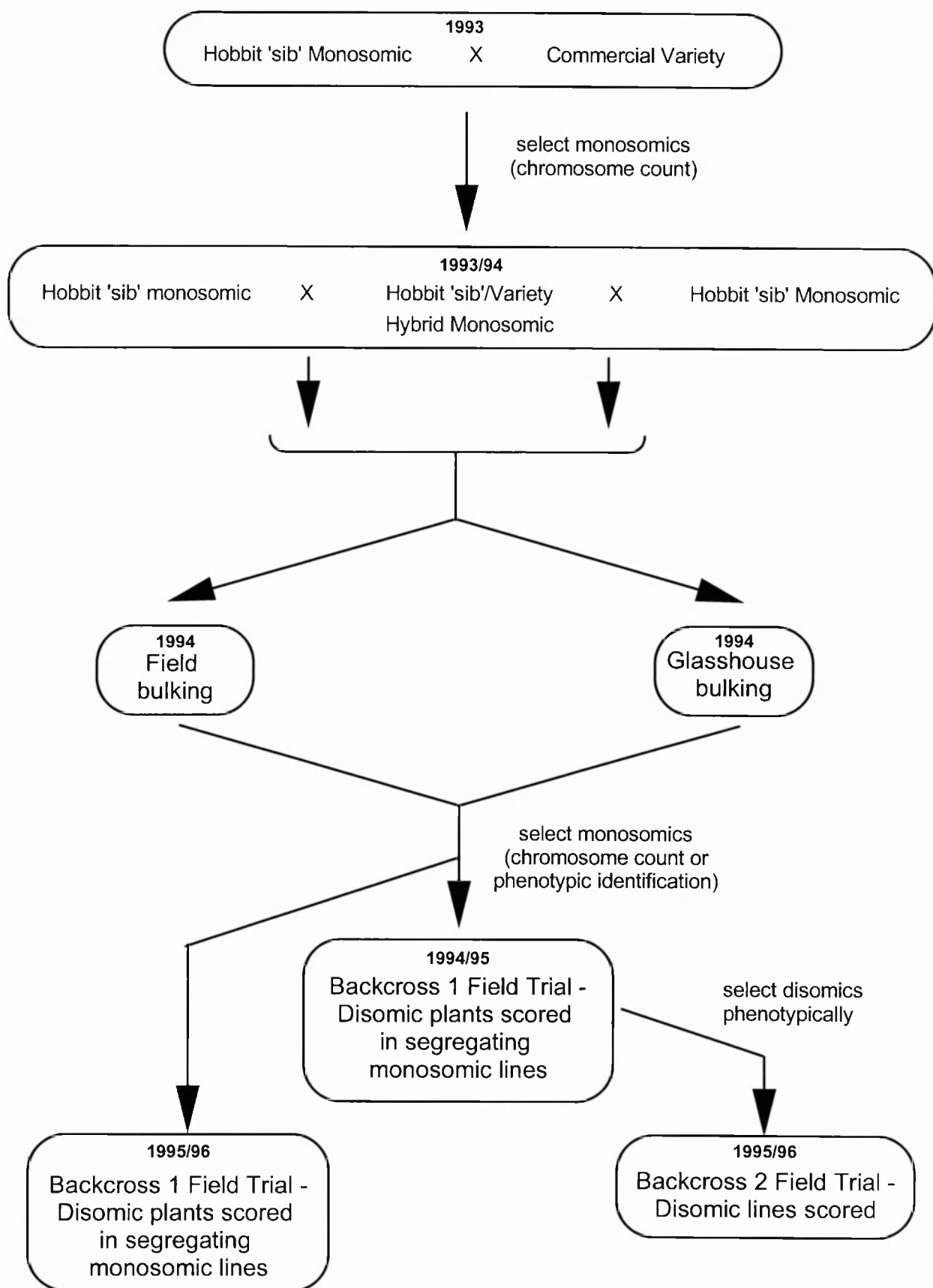


Figure 6.6: Generation of PBI commercial variety backcross reciprocal monosomic Lines, illustrating the key stages in the development of the lines.

progeny were selected from each cross for use in the development of the reciprocal monosomic lines. Monosomic plants were backcrossed reciprocally with the respective Hobbit 'sib' monosomics, as described in section 2.1.6.

Crossing

A number of problems were experienced during the crossing programme. Crossing was carried out in early January, so environmental conditions were not ideal for pollen production. There were also a reduced number of tillers on some plants. The combination of these factors led to low pollen production and seed set in some lines. The crosses carried out for each comparison and the number of seeds set are detailed in Table 6.4. Of the 211 crosses carried out over the 26 day crossing period, 142 crosses produced seed. Whilst some of the unsuccessful initial crosses can be attributable to inexperience, glasshouse conditions also played an important role in the success rate. The lights used in the glasshouse at the time were subsequently found to be faulty, resulting in low light intensity, hence the low seed set. Conditions appeared to affect some lines more than others. Problems were particularly experienced with the development of families involving monosomic chromosome 5BL-7BL. This monosomic was slightly necrotic and produced fewer tillers. There was very low production of pollen in the Hobbit 'sib' monosomic 5BL-7BL/variety lines. This factor was apparent for several of the 'first' backcross lines, as demonstrated by the fewer crosses and seed set when using the Hobbit 'sib' monosomic/variety lines as male parents. Due to the relatively short crossing period, relative to the number of lines involved, there were a limited number of repeat crosses that could be made if seed set had been low in initial crosses.

Due to low seed set, usually in the varietal reciprocal, 16 reciprocal families had insufficient seed to be further analysed and thus were removed from the experiment. A total of

Variety	Cross	No. of Crosses	Total No. of Seed Set
Genesis	Hobbit 'sib'/Genesis (3B Hobbit 'sib')	1	5
	Hobbit 'sib'/Genesis (3B Genesis)	1	4
	Hobbit 'sib'/Genesis (4B Hobbit 'sib')	2	14
	Hobbit 'sib'/Genesis (4B Genesis)	1	9
	Hobbit 'sib'/Genesis (4D Hobbit 'sib')	3	11
	Hobbit 'sib'/Genesis (4D Genesis)	2	9
	Hobbit 'sib'/Genesis (5BL-7BL Hobbit 'sib')	2	11
	Hobbit 'sib'/Genesis (5BL-7BL Genesis)	0	0
	Hobbit 'sib'/Genesis (5D Hobbit 'sib')	3	17
	Hobbit 'sib'/Genesis (5D Genesis)	1	8
Haven	Hobbit 'sib'/Haven (3B Hobbit 'sib')	3	15
	Hobbit 'sib'/Haven (3B Haven)	1	2
	Hobbit 'sib'/Haven (4B Hobbit 'sib')	2	4
	Hobbit 'sib'/Haven (4B Haven)	2	9
	Hobbit 'sib'/Haven (5BL-7BL Hobbit 'sib')	5	15
	Hobbit 'sib'/Haven (5BL-7BL Haven)	1	8
	Hobbit 'sib'/Haven (4D Hobbit 'sib')	2	11
	Hobbit 'sib'/Haven (4D Haven)	0	0
	Hobbit 'sib'/Haven (5D Hobbit 'sib')	1	8
	Hobbit 'sib'/Haven (5D Haven)	2	7
Hereward	Hobbit 'sib'/Hereward (3B Hobbit 'sib')	4	25
	Hobbit 'sib'/Hereward (3B Hereward)	2	8
	Hobbit 'sib'/Hereward (4B Hobbit 'sib')	5	14
	Hobbit 'sib'/Hereward (4B Hereward)	2	2
	Hobbit 'sib'/Hereward (5BL-7BL Hobbit 'sib')	2	25
	Hobbit 'sib'/Hereward (5BL-7BL Hereward)	0	0
	Hobbit 'sib'/Hereward (4D Hobbit 'sib')	2	14
	Hobbit 'sib'/Hereward (4D Hereward)	2	3
	Hobbit 'sib'/Hereward (5D Hobbit 'sib')	4	14
	Hobbit 'sib'/Hereward (5D Hereward)	1	4
Hornet	Hobbit 'sib'/Hornet (3B Hobbit 'sib')	3	25
	Hobbit 'sib'/Hornet (3B Hornet)	1	3
	Hobbit 'sib'/Hornet (4B Hobbit 'sib')	2	11
	Hobbit 'sib'/Hornet (4B Hornet)	2	14
	Hobbit 'sib'/Hornet (5BL-7BL Hobbit 'sib')	4	10
	Hobbit 'sib'/Hornet (5BL-7BL Hornet)	0	0
	Hobbit 'sib'/Hornet (4D Hobbit 'sib')	6	31
	Hobbit 'sib'/Hornet (4D Hornet)	1	4
	Hobbit 'sib'/Hornet (5D Hobbit 'sib')	3	17
	Hobbit 'sib'/Hornet (5D Hornet)	1	3
Hunter	Hobbit 'sib'/Hunter (3B Hobbit 'sib')	4	14
	Hobbit 'sib'/Hunter (3B Hunter)	1	2
	Hobbit 'sib'/Hunter (4B Hobbit 'sib')	2	18
	Hobbit 'sib'/Hunter (4B Hunter)	2	13
	Hobbit 'sib'/Hunter (5BL-7BL Hobbit 'sib')	2	7
	Hobbit 'sib'/Hunter (5BL-7BL Hunter)	2	3
	Hobbit 'sib'/Hunter (4D Hobbit 'sib')	3	25
	Hobbit 'sib'/Hunter (4D Hunter)	3	24
	Hobbit 'sib'/Hunter (5D Hobbit 'sib')	2	17
	Hobbit 'sib'/Hunter (5D Hunter)	0	0

**Table 6.4: Number of crosses made and total seed set for each reciprocal family.
The monosomic chromosome in each cross is shown in brackets.**

Variety	Cross	No. of Crosses	Total No. of Seed Set
Rialto	Hobbit 'sib'/Rialto (3B Hobbit 'sib')	4	7
	Hobbit 'sib'/Rialto (3B Rialto)	1	5
	Hobbit 'sib'/Rialto (4B Hobbit 'sib')	2	9
	Hobbit 'sib'/Rialto (4B Rialto)	3	12
	Hobbit 'sib'/Rialto (5BL-7BL Hobbit 'sib')	1	8
	Hobbit 'sib'/Rialto (5BL-7BL Rialto)	0	0
	Hobbit 'sib'/Rialto (4D Hobbit 'sib')	2	10
	Hobbit 'sib'/Rialto (4D Rialto)	1	7
	Hobbit 'sib'/Rialto (5D Hobbit 'sib')	3	16
	Hobbit 'sib'/Rialto (5D Rialto)	1	2
Riband	Hobbit 'sib'/Riband (3B Hobbit 'sib')	3	8
	Hobbit 'sib'/Riband (3B Riband)	1	5
	Hobbit 'sib'/Riband (4B Hobbit 'sib')	4	25
	Hobbit 'sib'/Riband (4B Riband)	1	6
	Hobbit 'sib'/Riband (5BL-7BL Hobbit 'sib')	1	2
	Hobbit 'sib'/Riband (5BL-7BL Riband)	1	8
	Hobbit 'sib'/Riband (4D Hobbit 'sib')	2	11
	Hobbit 'sib'/Riband (4D Riband)	1	12
	Hobbit 'sib'/Riband (5D Hobbit 'sib')	2	26
	Hobbit 'sib'/Riband (5D Riband)	2	2

 = Lines selected for bulking

Table 6.4: Continued

19 reciprocal families were taken through to the first bulking/field trial stage. These lines are highlighted in yellow in Table 6.4.

Bulking of Lines/Initial Field Trial Stage

The main aim at this stage was to provide individual bulked monosomic lines for each reciprocal family, each derived from single seeds. Of the 19 reciprocal families highlighted in Table 6.4, seed from 14 families was sown in a single plant randomisation in a field trial in 1994. Due to the potential risk of plant loss in the field, the 5 reciprocal crosses yielding small seed numbers (4 or 5 seeds per cross) were not sown, but bulked as single plants in a glasshouse. Two plants of each of the crosses sown in the field trial were also grown in the glasshouse as a backup against plant loss in the field trial. The results of the bulking stage are summarised in Table 6.5.

Environmental conditions caused problems for both plant establishment and development of yellow rust in the initial field trial. Due to the post-crossing harvest interval and an eight week vernalisation period, plants were not available for transplanting into the field until late April 1994. As a result, the plants were late developing and had a reduced tiller number. In addition, waterlogging of the field trial and damage by rabbits severely affected the establishment of some plant populations. The later flowering period of the plants coincided with the hot, dry conditions of the summer period and thus reduced grain filling potential. The combination of these conditions also restricted disease development, and prevented analysis of yellow rust resistance in the backcross monosomics. As a result of these environmental conditions, a number of plants were lost during the season, and the seed production of the remainder was reduced.

The progeny of the reciprocal backcrosses sown for bulking would have segregated for chromosome number, being predominantly either monosomic or disomic for the chromosome under test. Therefore, approximately 25% of the bulked lines would have been disomic. In

Variety	Chromosome	Method of bulk	Lines available for analysis
Genesis	Hobbit 'sib'/Genesis (3B Hobbit 'sib')	Glasshouse	3
	Hobbit 'sib'/Genesis (3B Genesis)		2
	Hobbit 'sib'/Genesis (4B Hobbit 'sib')	Field & Glasshouse	6
	Hobbit 'sib'/Genesis (4B Genesis)		4
	Hobbit 'sib'/Genesis (4D Hobbit 'sib')	Field & Glasshouse	2
	Hobbit 'sib'/Genesis (4D Genesis)		3
	Hobbit 'sib'/Genesis (5D Hobbit 'sib')	Field & Glasshouse	8
	Hobbit 'sib'/Genesis (5D Genesis)		5
Haven	Hobbit 'sib'/Haven (4B Hobbit 'sib')	Glasshouse	1
	Hobbit 'sib'/Haven (4B Haven)		1
	Hobbit 'sib'/Haven (5BL-7BL Hobbit 'sib')	Field & Glasshouse	6
	Hobbit 'sib'/Haven (5BL-7BL Haven)		5
	Hobbit 'sib'/Haven (5D Hobbit 'sib')	Glasshouse	1
	Hobbit 'sib'/Haven (5D Haven)		1
Hereward	Hobbit 'sib'/Hereward (3B Hobbit 'sib')	Field & Glasshouse	7
	Hobbit 'sib'/Hereward (3B Hereward)		4
	Hobbit 'sib'/Hereward (5D Hobbit 'sib')	Field & Glasshouse	1
	Hobbit 'sib'/Hereward (5D Hereward)		2
Hornet	Hobbit 'sib'/Hornet (4B Hobbit 'sib')	Field & Glasshouse	5
	Hobbit 'sib'/Hornet (4B Hornet)		7
	Hobbit 'sib'/Hornet (4D Hobbit 'sib')	Glasshouse	2
	Hobbit 'sib'/Hornet (4D Hornet)		2
Hunter	Hobbit 'sib'/Hunter (4B Hobbit 'sib')	Field & Glasshouse	8
	Hobbit 'sib'/Hunter (4B Hunter)		1
	Hobbit 'sib'/Hunter (4D Hobbit 'sib')	Field & Glasshouse	11
	Hobbit 'sib'/Hunter (4D Hunter)		1
Rialto	Hobbit 'sib'/Rialto (3B Hobbit 'sib')	Glasshouse	2
	Hobbit 'sib'/Rialto (3B Rialto)		3
	Hobbit 'sib'/Rialto (4B Hobbit 'sib')	Field & Glasshouse	3
	Hobbit 'sib'/Rialto (4B Rialto)		4
	Hobbit 'sib'/Rialto (4D Hobbit 'sib')	Field & Glasshouse	3
	Hobbit 'sib'/Rialto (4D Rialto)		2
Riband	Hobbit 'sib'/Riband (3B Hobbit 'sib')	Field & Glasshouse	3
	Hobbit 'sib'/Riband (3B Riband)		4
	Hobbit 'sib'/Riband (4B Hobbit 'sib')	Field & Glasshouse	6
	Hobbit 'sib'/Riband (4B Riband)		2
	Hobbit 'sib'/Riband (4D Hobbit 'sib')	Field & Glasshouse	2
	Hobbit 'sib'/Riband (4D Riband)		5

 = Lines analysed in 3 rep field trial (1994/95 & 1995/96)

Table 6.5: Number of lines available for field trials after bulking.
The monosomic chromosome in each cross is shown in brackets.

addition, approximately 3% of the progeny from the male monosomic parent crosses would be expected to be nullisomic, due to nullisomy of the male gametes. Therefore, a number of bulked lines would be unsuitable for further analysis due to segregation for disomy or nullisomy.

Where possible, monosomics were selected from both the glasshouse and field bulkings using phenotypic characters. The remainder were examined cytologically to identify monosomics. Families with four or more lines from each cross and with sufficient seed to sow a 3 replicate field trial were further analysed. The lines sown for bulking, and the number of lines available for analysis are detailed in Table 6.5. Of the 19 families entered into the bulking phase, only five families had sufficient lines and seed of both reciprocal crosses to be further analysed. As with the earlier crossing stage, whilst sufficient seed may have been available for one cross, a shortage of seed for the reciprocal cross meant the family had to be withdrawn from the analysis. The loss of plants was predominantly due to plant death, although segregation of disomics and nullisomics was responsible for some 'loss' of lines.

6.3.4 Field Trials

The five remaining families were tested for yellow rust resistance in 1994/95 and 1995/96. The five chromosomes analysed were 3B Hereward, 4B Genesis, 4B Hornet, 5BL-7BL Haven and 5D Genesis. "Backcross self 1" lines, the monosomic progeny of the bulking stage, were sown in 1994/95. These lines segregated monosomic and disomic plants. The disomic plants were selected phenotypically for disease scoring to ensure observed effects were due to chromosome effects rather than dosage effects. Disomic plants from these lines were selected and sown as disomic rows in 1995/96. The disomic lines made up the "Backcross self 2" populations. In addition, a further "Backcross self 1" field trial was carried out in 1995/96 using the remaining monosomic progeny from the bulking stage (see Fig. 6.6).

The backcross reciprocal monosomic lines were sown, together with Hobbit 'sib' and varietal controls, drilled as rows in 3 randomised, replicate blocks (Section 2.3.6). All trials were infected with the three race mixture 41E136(4), 169E168 and 237E141. Plants were scored for percentage flag leaf infection at GS 54 to 65 (Zadoks *et al.*, 1974).

6.3.5 Data Analysis

Reciprocal family means were calculated using the following formula:

$$\text{Mean} = \Sigma x/n$$

where x represents a 3 rep mean for each contributing line and n equals the number of test lines in each family.

The 'within sample' variance and standard deviation of each reciprocal family were calculated using the following formulae:

- (i) Sums of squares of deviations from sample mean

$$\Sigma d^2 = \Sigma x^2 - (\Sigma x)^2/n$$

- (ii) Variance

$$\sigma^2 = \Sigma d^2/n-1$$

- (iii) Standard deviation

$$\sigma = \sqrt{(\Sigma d^2/n)}$$

The standard deviation of the difference between two reciprocal means was calculated as:

$$\sigma_{\text{difference}} = \sqrt{((\sigma^2_{\text{variety}}/n_{\text{variety}}) + (\sigma^2_{\text{Hobbit 'sib'}/n_{\text{Hobbit 'sib'}}))}$$

where:

$\sigma^2_{\text{variety}}$ = variance of the reciprocal variety cross

$\sigma^2_{\text{Hobbit 'sib'}}$ = variance of the reciprocal Hobbit 'sib' cross.

n_{variety} = no. of observations for the reciprocal variety cross

$n_{\text{Hobbit 'sib'}}$ = no. of observations for the Hobbit 'sib' cross

The 5% Least Significant Difference (LSD) was calculated using t-values detailed in Fisher & Yates (1967) as:

$$\text{LSD}_{5\%} = t_{d.f} \times \sigma_{\text{difference}}$$

6.3.6 Results

Parental Scores

Parental flag leaf scores for the three experiments over the two seasons are shown in Figure 6.7. The results show that there were clearly strong resistance effects present in certain varieties relative to Hobbit 'sib'. Although the race mix used should be virulent to most of the major gene complexes present in the varieties, it is possible that certain major genes were not overcome. This is almost certainly the case in varieties such as Hunter, where no race has yet been identified to overcome its resistance complex. There may also be residual resistance effects present in the varieties that are not attributable to major genes but that prevented significant development of the disease.

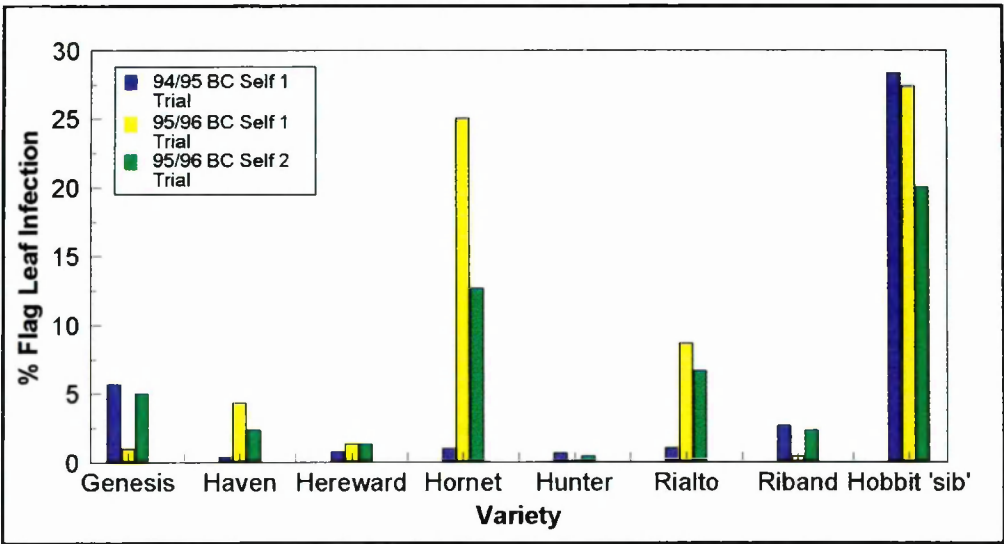


Figure 6.7: Parental flag leaf yellow rust infection scores

The change in disease level on Hornet between the 1994/95 and 95/96 season clearly indicated a race effect. It is likely that a change in race balance between the two years resulted in this change. The increase in disease level in the second year would suggest that a race virulent to Hornet did not become properly established in the first year. An apparent but small increase in disease level on Rialto and Haven was also noted. Races 237E141 is virulent to these varieties. It is possible that this race did not establish properly in the first year of trials.

The variation in disease levels, and the apparent presence of significant resistance effects in a number of varieties will have an important effect on the analysis of the backcross reciprocal monosomic lines. If these effects are located on the chromosomes under investigation, the effect should be clearly noticeable. If the effects are located on the background chromosomes, they should segregate within the families and thus may give a large 'within family' standard deviation.

Backcross Self 1 Results

Five reciprocal comparisons were tested in 1994/95 and 1995/96. Disomic plants were selected phenotypically from the segregating population at GS 50 to 54 (Zadoks *et al*, 1974), and scored for flag leaf yellow rust infection.

1994/95 Trial

Problems were experienced identifying disomic plants, as the plants were too closely spaced, and in some cases growing together. This made distinction of individual plants, and thus the differentiation between disomic and monosomic plants difficult. As a result, disomics were only identified in four or more individual lines/family for one comparison, namely 5BL-7BL Haven (Table 6.6). The mean scores of the two families showed very little difference between each other, and the differences were considerably less than the 'LSD' scores.

Variety	Chromosome	Score	Hobbit 'sib'			Variety			No. of lines	SD	Mean	SD	No. of lines	Difference of means	LSD (5%)
			No. of lines	Mean	SD	No. of lines	Mean	SD							
Genesis	4B	1995/96-1	5	8.27	12.56	4	2.89	2.83	4	5.39	13.71	5.39	4	5.39	13.71
		1995/96-2	5	7.55	13.49		2.13	1.02							
	5D	1995/96-1	5	18.77	11.26	5	11.77	8.16	5	7.00	14.33	7.00	5	7.00	14.33
		1995/96-2	5	13.70	14.53		9.91	5.14							
Haven	5BL-7BL	1994/95-1	5	6.63	8.82	4	9.00	10.20	4	2.37	14.05	2.37	4	2.37	14.05
		1994/95-2	5	5.56	5.45		10.80	9.75							
	5BL-7BL	1995/96-1	6	11.87	9.04	4	7.83	6.78	4	4.04	11.56	4.04	4	4.04	11.56
		1995/96-2	6	8.72	9.56		13.25	13.29							
Hereward	3B	1995/96-1	7	8.91	9.69	4	2.56	1.88	4	6.35	8.56	6.35	4	6.35	8.56
		1995/96-2	7	10.00	6.43		3.81	5.48							
Hornet	4B	1995/96-1	5	12.60	7.82	6	12.17	10.69	6	0.43	12.66	0.43	6	0.43	12.66
		1995/96-2	5	15.67	14.55		13.86	18.32							

Table 6.6: Comparison of mean flag leaf infection levels for backcross self 1 disomic families, showing the number of lines, the mean and standard deviation for each 'Hobbit sib' and 'varietal' family. Two scores are detailed for each cross for the 1995/96 season, with an additional 1994/95 season score for one family. The 5% Least Significant Difference values show that none of the reciprocal family means are significantly different. 1994/95 Hobbit 'sib' control mean = 28.33% flag leaf infection. 1995/96 Hobbit 'sib' control mean = 23.66% flag leaf infection.

The reciprocal comparisons for the remaining 4 families lacked sufficient lines to carry out a reliable analysis. For the 3B Hereward, 4B Genesis and 5D Genesis families, whilst disomics were identified in four or more of the Hobbit 'sib' specific lines, no more than three lines containing disomics were identified for the reciprocal varietal cross. The 4B Hornet family lacked disomic scores for two Hobbit 'sib' specific lines. Mean comparisons for these four comparisons therefore lacked contributing lines and were considered unreliable due to potential bias from the remaining lines.

1995/96 Results

Accurate plant spacing was achieved in the repeat trial by sowing with a more precise, Wintersteiger 'Spider' drill. This facilitated easier identification of disomic plants. Disomic plants were identified and scored in four or more lines/family for all five families tested (Table 6.6). However, in four of the five comparisons, disomic plants were not identified in all 3 replicates of the reciprocal families. Family means for 3B Hereward, 4B Genesis, 4B Hornet and 5D Genesis were therefore non-orthogonal. Each family mean was made up of a number of individual line means; each of which was a 3 replicate mean. It is possible that a lack of replication would introduce bias into the results. The lines were still segregating and demonstrated a large level of variation in some families, as indicated by the large 'within family' standard deviation. For example, both the Hobbit 'sib' and Hornet families in the 4B Hornet comparison had large standard deviations and hence LSDs, particularly at score 2. This deviation is predominantly due to the single replicate scores introducing bias to the family means. These results are therefore unreliable and firm conclusions can not be made about these four reciprocal families.

There were a sufficient number of lines in the 5BL-7BL Haven analysis, with disomics identified in all three replicates. However, there was no significant difference between the family

Variety			Hobbit 'sib'			Variety			No. of lines	SD	Mean	SD	No. of lines	Difference of means	LSD (5%)
			Chromosome	Score	No. of lines	Mean	SD	Mean							
Genesis	4B	1995/96-1	8	9.18	8.85	7.86	6.22	9	1.32	8.00					
		1995/96-2	8	9.30	9.22	11.20	6.13	9	1.90	8.20					
	5D	1995/96-1	9	9.15	6.63	15.20	8.11	10	6.05	7.14					
		1995/96-2	9	12.70	7.94	16.20	7.86	10	3.50	7.67					
Haven	5BL-7BL	1995/96-1	10	9.87	4.44	6.04	5.87	10	3.83	4.89					
		1995/96-2	10	15.40	6.11	9.93	7.66	10	5.47	6.51					
Hereward	3B	1995/96-1	10	10.60	8.08	5.48	4.51	7	5.12	6.55					
		1995/96-2	10	16.40	7.26	16.00	11.51	7	0.40	10.48					
Hornet	4B	1995/96-1	7	15.00	9.81	17.50	13.40	9	2.50	12.32					
		1995/96-2	7	18.30	7.72	19.90	12.81	9	1.60	11.10					

Table 6.7: Comparison of 1996 score 1 and score 2 mean flag leaf infection levels for backcross self 2 disomic families, showing the number of lines, the mean and standard deviation for each 'Hobbit sib' and 'varietal' family.
The 5% Least Significant Difference values show that none of the reciprocal family means are significantly different.
1995/96 Hobbit 'sib' control mean = 23.66% flag leaf infection

means.

Backcross Self 2 Results

Although identification of disomic plants in the field in the 1994/95 season had proved difficult, harvesting and separation of whole individual plants enabled the identification of additional disomic lines. These were planted in a field trial in 1995/96, and scored as whole lines at Growth stage 54 to 65. However, it was not possible to identify disomics for four or more lines in the variety specific families of 3B Hereward and 4B Genesis. Sufficient disomic families were identified to test chromosomes 4B Hornet, 5BL-7Bl Haven and 5D Genesis. No significant differences between family means were identified for any of the 3 chromosome families tested (Table 6.7).

6.3.7 Discussion

A key requisite of the backcross reciprocal technique was to derive enough independent lines within each family to ensure that on average the backgrounds will be the same, essentially a backcross between the tester line (Hobbit 'sib') and the variety. Thus, any variation between reciprocal families could be attributed to the chromosome under examination. The lack of lines in the majority of the reciprocal crosses prevented subsequent analysis, as an 'average' genetic background could not be ensured. Sufficient lines were available for only a small number of disomic families (5BL-7BL Haven in 94/95 and 95/96; 4B Hornet and 5D Genesis in 95/96). However, for each of the variety chromosomes examined, no significant differences were detected between the Hobbit 'sib' chromosome and the varietal chromosome. The null hypothesis can therefore be accepted. This suggests that the varietal chromosomes carry the same genes for susceptibility or genes of similar effect as Hobbit 'sib' on the chromosomes examined.

Examination of the parental scores shows six of the seven varieties to be considerably

more resistant to yellow rust than Hobbit 'sib' (Fig. 6.7). This suggests the presence of genes for resistance in the varieties that have not been overcome by the yellow rust race combination. The use of three races was intended to overcome all known major resistance genes in the parental varieties. However, a number of varieties, such as Genesis, were less infected than expected. This suggests that not all three race isolates became established to the same level. It would appear that race 41E136(4) was fully established, giving levels of disease on Hobbit 'sib' similar to single race trials (Chapter 3). However, the other races did not cause the expected level of disease on the commercial varieties. It is possible therefore that some known major resistances remained effective. In addition, some varieties may have contained unknown major resistance genes or strong background resistance that would not be overcome by the races. Background resistance has also been demonstrated in the Hobbit 'sib' monosomics on chromosomes 1A, 2A, 2B, 2D, 4A, 5BS-7BS and 6D, including Yr16 on 2D and un-named resistance genes on 5BS-7BS, that may be present in some reciprocal families.

If these resistance genes were located on the chromosomes examined then the reciprocal family containing the varietal hemizygous chromosome could be expected to be more resistant than the reciprocal Hobbit 'sib' family. The similarity in disease level for the two families in each case examined would therefore suggest that any different resistance genes in the varieties must be located on the background chromosomes, and will therefore be segregating in the reciprocal families. The segregation of strong resistance effects in the background of the families will therefore increase the variation and standard deviation of the populations. Thus any calculation of the least significant difference between reciprocal families will be large, and significant differences between reciprocal families will be difficult to detect. As such, the null hypothesis might be accepted by default.

As the results have demonstrated, the wide variance of the reciprocal families has made subsequent rejection of the null hypothesis difficult. The apparent similarity between the effects of homologous chromosomes may in fact be due to a number of reasons. Background variation may have a larger effect than the difference between reciprocal chromosomes and thus mask the chromosome effects. Alternatively, the effect of the chromosomes may be identical, but due to different genetic factors. For example, one chromosome carrying a weak resistance gene and one carrying a weak suppressor may have a similar overall resistance level. Therefore, the null hypothesis can only be used to demonstrate significant differences between chromosomes.

A revised null hypothesis is necessary in order to test for similarity. Demonstration of a lack of difference could be used as the first stage. Furthermore, a similar change in disease level between monosomy and disomy would demonstrate that a varietal chromosome has a similar effect to the Hobbit 'sib' homologue. However, as demonstrated by Pink *et al* (1983) in the Chinese Spring group 5 chromosomes, the net effect of the chromosomes may be due to the balance of genes for resistance and genes for susceptibility. Therefore, despite similar chromosomal effects, the gene balance of the individual chromosomes may be different. To test for genetic similarity, a segregational test for gene allelism between the two chromosomes is necessary, as was carried out to test for allelism between the Bezostaya chromosomes and their mutant homologues.

Future Work

The time constraints involved in the project, both in terms of the length of the total project, and also the restricted 'crossing window' available to set up the original populations resulted in a shortage of lines per family. In addition, large losses at the bulking stage reduced the number of lines yet further. This has caused problems in both the number of varietal

chromosomes that could be tested, and the reliability of the data from those that were tested. The large background variation component of the experiment was greater than expected. As such, differentiation between family means was statistically non-significant. A greater number of lines would reduce the effect of outlying disease values, and reduce the standard deviation of the populations giving a better estimation of the population mean. This would enable easier distinction between reciprocal means. A large number of monosomic lines are probably needed per family to account for background variation in order to achieve an 'average' genetic background. In addition, to ensure satisfactory bulking of the lines, the F1 backcross plants should be bulked in the glasshouse to reduce their risk of plant loss due to adverse environmental conditions. As the generation of a larger number of lines per family is a priority requirement, concentration on fewer varieties or chromosomes would be recommended. This would enable more lines to be produced per reciprocal, as effort could be concentrated more effectively on fewer lines.

The backcross reciprocal monosomic technique was used by Buerstmayr (1997) to study head blight resistance in wheat, again using Hobbit 'sib' as the susceptible parent. He concentrated on only two varieties, but studied all 21 chromosomes as a single experimental project for a 3 year thesis. Two crossing seasons were used to generate the lines, followed by a further to multiply the families. As a result, only one field trial season was available in the equivalent time block. However, an increased number of lines were generated, ranging from 6 to 15 lines per family. Thus, the results were more reliable. He noted significant differences between the varieties on several chromosomes, where the Hobbit 'sib' line was more susceptible. This indicated either that the varietal chromosomes carried a gene for resistance, or lack a gene for susceptibility present in Hobbit 'sib'. In one variety, the varietal chromosome 2B was more

susceptible than Hobbit 'sib', indicating the presence of a gene for susceptibility in the variety that is not present in Hobbit 'sib'. Whilst the generation of a greater number of lines enabled the identification of a number of chromosomes exhibiting differences, the effects of the majority of the homologous chromosome pairs did not differ from each other. As with the results above, this could not be used as an indication of similarity between the two head blight resistant varieties and Hobbit 'sib'.

A reduction in the number of varieties investigated would make disease testing and race deployment easier. With fewer major gene combinations to contend with, it may be possible to use race mixtures specific to more of the major genes involved, thus reducing their influence on background variation. Separate trial blocks could also be used for each variety, to reduce the number of races used in each mixture and thus possibly reduce race competition.

A backcross reciprocal comparison of Bezostaya with Hobbit 'sib' would provide a useful evaluation of the technique for investigating disease resistance. The evaluation of the Hobbit 'sib'/Bezostaya substitution lines by Worland & Law (1991) provides valuable data on the relative effects of each of the chromosomes. A comparison of the reciprocal monosomic results with the substitution line data would therefore give an indication of how reliable the reciprocal monosomic analysis method was for detecting chromosome effects for a polygenic character such as yellow rust resistance.

6.4 General Discussion

Both techniques for determining the presence and location of genes for susceptibility in other varieties have proved difficult to use and rather time consuming. The complexity of the control of adult plant resistance, including the involvement of several chromosomes makes precise analysis difficult. The Hobbit 'sib'/Bezostaya substitution lines clearly demonstrate the usefulness of substitution line analysis in identifying chromosomes affecting disease resistance. However, more detailed analysis of these lines to test for deletion mutant allelism has proved more difficult. Furthermore, a long timescale is needed to develop the substitution lines. Backcross reciprocal monosomic analysis was used in an attempt to reduce this timescale, but problems have been experienced using this method due to the large number of factors involved in the control of the resistance response.

A more direct way of determining the presence of genes for susceptibility in varieties may be to analyse F₂ segregating populations of crosses between the varieties of interest and deletion mutants, as demonstrated in Chapter 5. This requires identification of deletions linked to resistance before conclusions can be drawn, but if this is possible, F₂ segregation analysis may achieve both identification of putative susceptibility alleles, and markers for those alleles in one process. If a deletion causal for resistance can be demonstrated to segregate with resistance in an F₂ population, it would suggest that the variety of interest also contains the gene for susceptibility identified in Hobbit 'sib'. The deletion may also act as a marker for that susceptibility allele, enabling the removal of that allele in a breeding programme.

CHAPTER 7

GENERAL DISCUSSION

7. General Discussion

Putative genes for susceptibility have been identified in Hobbit 'sib' (Worland & Law, 1991). Monosomic analysis of Hobbit 'sib' enabled the identification of genetic factors that reduced the resistance of Hobbit 'sib'. It was hypothesized that the alteration or removal of these putative genes for susceptibility by means of induced mutation would improve resistance to yellow rust. Mutation has been shown to improve disease resistance in a number of systems (Williams *et al*, 1992; Wiberg, 1973; Mullins, 1995). A good example of this is the mutation to resistance of the gene determining susceptibility to stem rust on chromosome 7D in the wheat variety, Canthatch (Kerber, 1991). It should therefore be possible to achieve similar results in Hobbit 'sib'.

7.1 The induction of resistance via mutation

The irradiation of Hobbit 'sib' with fast neutrons to induce deletions in genes for susceptibility produced several mutants with increased resistance to a number of diseases. Further screening of these mutants in this study confirmed increased resistance to yellow rust in 17 mutants. Whilst it is hypothesized that Hobbit 'sib' would normally carry a balance between opposing sets of genes for resistance and susceptibility, the results indicate inactivation of genes for susceptibility by mutation alters the overall balance making adult plants more resistant. The levels of resistance differ between the mutants, indicating either that different genes for susceptibility have been affected in the individual mutants, or the balance between genes for susceptibility and resistance has been differentially affected in individual mutants.

Additional evidence on the importance of being able to eliminate genes that reduce resistance levels was obtained by screening the yellow rust resistant mutants to attacks of brown rust and mildew. The results showed that a small number of mutants were also resistant to brown

rust or mildew, and in the case of I3-48, to all three diseases. This supports the findings of Pink *et al* (1983), where altering the dosage of group 5 chromosomes simultaneously changed the plants ability to resist infection by both yellow rust and mildew. It was proposed that if resistance to both diseases is determined by the same locus, that locus might be considered to determine a basic compatibility between host and pathogen.

Correlation of disease resistance may be due to two possibilities. First, susceptibility to yellow rust and either or both of the other two diseases may be due to a single locus; the solution suggested in the previous paragraph. Mutation at this locus would produce a correlated effect on resistance to several diseases. Alternatively, susceptibility to each disease may be due to separate but closely linked loci. Multiple mutations at these loci, or possibly a single deletion spanning these loci would account for the correlated disease resistance.

It is important to note that of the mutant lines showing correlated resistance to two or three disease, only one was located to group 5 chromosomes using monosomic analysis (Worland & Law, 1991). For example, monosomic analysis located the resistance of I3-48 to 4D. Deletion mapping identified a deletion on 4DL (Chapter 4) that subsequently was shown to be linked to both yellow rust and brown rust resistance (Chapter 5). The non-specific action of adult plant genes is, therefore, not confined to those loci on homoeologous group 5 chromosomes. Whilst no group 7 genes reducing the plants ability to withstand adult plant infection by various foliar diseases were detected in Hobbit 'sib', studies by Kerber & Green (1980) and Dyck (1987) suggested the presence of similar genes for susceptibility on chromosome 7D that, when removed, increased the resistance to several diseases.

7.1.4 Resistance to the Septoria diseases

No significant improvement in resistance was detected for either *Stagonospora nodorum*

or *Septoria tritici*. Significant increases in susceptibility to both diseases were detected in a few mutants. Some of these effects may be due to physical changes in the mutant, such as height differences, where shorter plants were more susceptible, whilst other effects may be due to mutation of resistance genes. The lack of improved resistance may either suggest that genes for susceptibility to either disease are not present in Hobbit 'sib', or, because selection was only carried out for yellow rust resistance, mutants showing genetic resistance to the Septorias were missed. If the latter is true, it is necessary to explain the appearance of brown rust and mildew resistance amongst the lines when selection was only practised for yellow rust, and not *S. nodorum* or *S. tritici* resistance.

Possibly some fundamental difference between the biotrophic and necrotrophic pathogens affects the likelihood of achieving resistance by genetic deletion. Biotrophs are much more reliant on the living host and rely on a constant supply of nutrients from the host to the growing fungus. This interaction may be more readily affected by mutation than the hemibiotrophic or necrotrophic interaction. Alternatively, genes for susceptibility to yellow rust, brown rust and mildew may be clustered together at different points in the genome to those affecting the necrotrophs. Therefore selection of deletion mutants with resistance to yellow rust may also select for brown rust and mildew resistance but deletions improving *Septoria* resistance are not selected as they are not linked to the 'yellow rust' deletions.

7.2 Mapping of deletions

Using a combination of RFLP and RDA markers, deletions have been identified in 12 of the 21 mutants studied. Amongst those 12 mutants, deletions have been identified on 9 different chromosomes, summarised in Fig. 4.1 and Table 4.1.

The initial rationale for using fast neutron radiation as a mutation agent was to produce

a relatively small number of deletions, that would be mostly small in size. (C.N. Law, pers. comm.). However, it is clear from these results that radiation produced a large number of deletions, distributed across the chromosomes examined. A number of mutants carry several deletions, located on several chromosomes. It is probable that these relate to several independent deletion events occurring in each of the mutants. Some of these deletions, such as the 5DL deletion in I3-32 are also large. Interestingly, fast neutron radiation has also been shown to produce a number of large deletions in *Arabidopsis thaliana* (Bruggemann *et al*, 1996). It can therefore be concluded that fast neutron radiation is likely to cause a large number of deletions, some of which are also large in size.

The large number of deletions has implications for the mutant phenotypes. It is unlikely that all deletions will be linked to resistance, as confirmed by the work detailed in Chapter 5. It is possible that some of these may be deleterious, or at the very least have adverse effects on agronomic performance. If mutants are to be used as a source of resistance, it would be useful to tag and screen as many deletions as possible in order to maintain resistance-linked deletions and remove potential deleterious deletions. A crucial aspect of detecting and mapping useful or deleterious deletions is to ensure a comprehensive coverage of the genome using available marker sets. The Cambridge Laboratory RFLP marker set, used for the RFLP analysis detailed in Chapter 4, has a number of 'gaps' in its coverage. There tend to be more markers clustered around the centromere than at the distal ends of the chromosomes (Devos *et al*, 1995). Therefore, future screening could be carried out with other marker sets to ensure a more comprehensive coverage of the genome. RFLP markers from the Opata 85 x Synthetic wheat map (Nelson *et al*, 1995) provide a better coverage of the wheat genome, with less clustering around the centromere than the Cambridge Laboratory RFLP set. Other marker systems such as microsatellites may also

provide additional markers to fill the gaps.

The same loci were found to be deleted in a number of independently derived mutant lines. This may be due to the effect of these deletions on resistance so that selection would favour their appearance in a number of mutants. Alternatively, certain parts of the genome may be more prone to deletion. This may be due to structural or sequence differences making certain regions of the genome more sensitive to deletion. The degree of methylation may also affect the likelihood of deletion. Methylation is thought to confer increased structural stability to chromosome regions (Matassi *et al*, 1992), and therefore may affect the probability of a deletion event occurring.

Representational Difference Analysis (RDA) (Lisitsyn *et al*, 1993) was used successfully to target deletions. Deletion mutants provide a very good starting material for RDA. Where the two genomes compared are identical except for the deleted sequences, then clones mapping to deletions should be relatively easily detected using RDA. RDA, whilst biased in terms of the mutant being studied, is not restricted to certain chromosomes and may isolate markers mapping to deletions in a number of mutants, potentially on any chromosome. The results of the mutant RDA analysis support the findings of the RFLP screening. Common deletions were detected in a number of mutants. Certain mutants, such as I3-54, were shown to contain several deletions on different chromosomes. Therefore, RDA analysis should be targeted in order to identify specific deletions. The use of the basic mutant as a driver genotype is likely to lead to the isolation of several clones for different deletions that are not necessarily linked to resistance. If the probable chromosome location of a deletion conferring resistance is known, as was the case for I3-54, where monosomic analysis identified a 4B location, the use of backcross monosomic lines containing only the chromosome of interest derived from the mutant should provide a more

targeted selection of deletion specific clones. Clones specific to 'background' deletions are then less likely to be isolated.

7.3 Segregation of deletions with resistance

Analysis of the segregation of deletions with resistance identified only one deletion that is possibly causal for yellow rust resistance. The 4DL deletion on I3-48, tagged by Xpsr 104 and Xpsr 163, showed complete segregation with resistance to yellow rust at F2. However, analysis of the segregation of this deletion with resistance to brown rust at the F3 produced two putative recombinants out of 69 that were susceptible to brown rust but were also homozygous for the deletion, indicating that the deletion was linked to the brown rust resistance but not responsible for it. Since an F3 segregation test for yellow rust resistance was not completed because of poor establishment of the disease in the field, the deletion could not be conclusively proven to be causal for yellow rust, as potential recombinants would not be identified until F3.

Depending upon whether the 4DL deletion is causal or linked to yellow rust resistance, a number of explanations for resistance to the two diseases are possible. If the deletion is causal for yellow rust resistance, then this suggests that the deletion has inactivated a gene for susceptibility to this disease. The 4DL deletion markers, Xpsr 104 and 163, are therefore effectively markers for a gene for susceptibility to yellow rust. The linkage of these loci to a factor conferring brown rust resistance suggests that there are at least two closely linked genes for susceptibility; one of these has been deleted to give yellow rust resistance and the other has either been involved in a separate deletion or has been inactivated to give brown rust resistance. Alternatively, a mutation close to the identified deletion has resulted in the formation of a brown rust resistance gene, rather than the inactivation of a gene for susceptibility.

However, if the deletion is not causal for resistance to either disease, but is closely linked

to them both then it is possible that resistance to both diseases is conferred by the same locus. This may be due to mutation of a component of the basic pathogen-host interaction, as discussed by Pink *et al* (1983). Alternatively, the common loci may represent suppressors of a locus showing resistance to more than one disease. A resistance gene with specificity for two different avirulence genes, RPM1 and RPS3, has been identified in *Arabidopsis* (Bisgrove *et al*, 1994). If a similar locus exists and is being suppressed in Hobbit 'sib', a single mutation in the suppressor of that loci may confer resistance to two different diseases.

The current results suggest that correlated resistance in I3-48, rather than being a product of a single broad spectrum locus, is probably due to a cluster of mutated genes for susceptibility. The 4DL deletion may therefore represent just one of several deletions or mutations in a cluster of genes affecting resistance. Further screening with markers in the region of the 4DL deletion will help to clarify the position and may identify further deletions in that area that are causal for resistance to brown rust.

Identifying genes for susceptibility using homoeologous relationships

Once a deletion causal for resistance has been identified in one mutant, it may be possible to predict the locations of other deletions for genes for susceptibility using the principle of homoeologous relationships between genes of common function. For example, recent studies have shown that the homoeologous Ppd genes, determining photoperiodic response, map to similar positions on the group 2 chromosomes compared to common RFLP markers and that this relationship is so strong that comparisons can be made right across the *Triticeae*, with PpdH1 of barley, for instance, mapping in a similar position on 2H to the wheat Ppd genes in comparison to common RFLP markers (Laurie *et al*, 1994).

A similar relationship may occur for genes for disease susceptibility. The presence of a

deletion conferring resistance in one mutant may identify markers useful for detecting deletions in genes for susceptibility on homoeologous chromosomes in other mutants.

Reduced transmission

In nearly all instances, the transmission of the deletion was reduced. A similar observation was made with fast neutron-generated mutants in *Arabidopsis thaliana*, where mutant alleles were poorly transmitted through the male gametophyte (Bruggemann *et al*, 1996). Segregation analysis is therefore essential to establish both useful and superfluous deletions. Reduced transmission of unlinked deletions also indicates that they may be deleterious to the mutant. Deletions may be affecting a physical aspect of the mutant, such as pollen viability, or possibly interfering with processes at the intracellular level, such as meiosis. Markers identifying non-linked deletions are therefore useful if these mutations need to be removed from breeding populations. Removal of these deletions will be facilitated by the lower transmission frequency, so it may not actually be necessary to use a marker system too extensively. If the deletions confer a selective disadvantage, it is likely that they will be removed from a breeding population as a matter of course.

Cleaning up deletion mutants

In terms of deletions conferring resistance to a susceptible host, the ideal scenario would be to obtain the parental line, with only the deletion(s) causal for resistance, but no deleterious background deletions. The first stage is obviously to identify the causal deletion and map it. Substitution lines in the susceptible parent could then be used to isolate the causal deletion in a background lacking the deleterious deletions. This could be done by constructing substitution lines with the relevant chromosome derived from the mutant. This technique has been described in chapter 2.1.4 (illustrated in Fig. 2.2). This will result in a line with a Hobbit 'sib' background, but with the chromosome containing the deletion conferring resistance.

A recombinant exercise could then be carried out to reduce the proportion of the mutant genome present in the substituted chromosome. If the substitution line is crossed to Hobbit 'sib', followed by subsequent selfing of the progeny, recombinant lines will be obtained. Selection of the Hobbit 'sib' phenotypes, also containing the deletion should ensure resulting lines that are close to the parental variety, but contain the deletion conferring resistance.

Ultimately, it would be preferable to introduce the deletion into modern cultivars to improve resistance. Direct introduction of Hobbit 'sib' derived mutations into modern cultivars such as Rialto has proved rather unsuccessful (W. van-der-Schaar, pers. comm.), most likely due to additional deletions having an adverse effect on the progeny. Selected Rialto/I3-48 lines have been taken through a number of additional generations for further general selection at PBI. However, although resistance to yellow rust has been successfully maintained, the vigour and performance of the plant is much reduced as well as being susceptible to *Septoria tritici*. Whilst this may be due to the deletion responsible for yellow rust resistance, it is probable that there are other deletions in the background that may also be having an effect. Therefore, it would be better if the useful deletion could be introduced without the problems associated with the background deletions.

Development of a monosomic series in a modern variety could be run in tandem with substitution line development, as was carried out with the development of the Hobbit 'sib' monosomics and Bezostaya substitution lines. This would provide a monosomic series so that useful mutations could easily be introduced into a modern cultivar background as they were detected, hopefully reducing the potentially deleterious effects of background chromosomes.

However, it should be considered that the deletions responsible for conferring resistance to the mutants may well have deleterious effects associated with them, either due to the deletion

of additional beneficial genes contained within the deletion, or possibly as a pleiotropic effect of alteration of expression of the gene conferring resistance. Therefore, introduction of deletions conferring resistance into modern varieties may not be desirable. It may be preferable to use the deletions to identify sections of chromosomes in other varieties that do not promote susceptibility, as illustrated by the allelism work reported in Chapter 6. The introduction these chromosome segments into modern cultivars would be less likely to have a deleterious effect than the insertion of deletions.

7.4 Distribution of genes for susceptibility amongst commercial varieties

Previous work has clearly demonstrated that there are differences in the distribution of genes for susceptibility or suppressors of resistance between varieties (Kerber, 1983). For example, the transfer of the Canthatch 7D resistance determining mutation into Chinese Spring (Kerber & Aung, 1995) does not confer resistance to the recipient variety. This suggests that Chinese Spring either has no suppressed resistance genes, or alternatively lacks the 7D suppressor identified in Canthatch. The identification of genes for susceptibility in modern varieties would provide a target for improvement of disease resistance, and details of their distribution could prove useful in breeding programmes.

The use of backcross reciprocal monosomic analysis to determine the distribution of genes for susceptibility in commercial varieties has not been successful. Reciprocal monosomic analysis has been successful previously to investigate characters such as final plant height (Snape & Law, 1980). However, the complex control of adult plant resistance, involving numerous genes on several chromosomes has made the analysis using this technique difficult. The large environmental component of the variation was also an important contributory factor in making the analysis less precise. To overcome these difficulties, many more F3 lines are needed if the

analysis is going to achieve any success. A number of methods to improve the reciprocal monosomic analysis technique for this application have been discussed in Chapter 6.

It would be useful to be able to identify genes for susceptibility using a relatively quick method. Techniques such as the development of substitution lines are very useful for detecting variation between varieties, but are very time consuming. A faster method is therefore desirable.

One method would be to use deletions known to confer resistance. Crosses between resistant mutants and commercial varieties could be tested at F2 for segregation of the causal deletion with resistance. If the deletion segregates with the resistant progeny, it could be assumed that the deletion confers resistance and that the commercial variety contains the same gene for susceptibility as that deleted in the mutant. However, this technique is limited in that it can only be used in varieties where the mutant is more resistant and can be readily distinguished from the variety. It can not be used to test resistant varieties, such as Hunter, as the effect of the deletion will not be detectable. The analysis is also limited to those assessing genes for susceptibility that have already been mapped in deletion mutants.

Ultimately, although a more time consuming and long term approach, the development of commercial variety chromosome substitution lines in Hobbit 'sib' may be necessary. A substitution line series would allow direct comparison of the variety and Hobbit 'sib' chromosomes.

7.5 The expression of mutation induced resistance

It has been clearly demonstrated that mutation has improved disease resistance in certain lines. It would therefore be interesting and informative to establish how this resistance is mediated. The host defense response has clearly been altered, enhancing resistance to a number of diseases. The study of pathogen-related (PR) protein expression in the mutants may give an

indication of how resistance has been altered.

The analysis of PR protein expression has been used previously to understand the effect of mutation-induced resistance in *Arabidopsis thaliana*. A number of mutants have been isolated in *Arabidopsis* with increased resistance to *Pseudomonas parasitica* (Dietrich *et al*, 1994; Bowling *et al*, 1994; Greenberg *et al*, 1994). Systemic Acquired Resistance (SAR) is a non-specific defense response, induced under normal circumstances in response to initial pathogen attack, that inhibits further attack by any pathogen. It was noted that SAR resulted in an elevated expression of PR proteins and an increase in the endogenous level of salicylic acid. It has been proposed that the mutations in *Arabidopsis* alter the regulation pathway of SAR, leading to constitutive expression of PR proteins. For example, the *cpr1* mutant constitutively expresses PR 1, and it is proposed that the *CPRI* gene acts as an upstream regulator of SAR, and that mutation removes that regulation (Bowling *et al*, 1994).

Whilst SAR has not been convincingly demonstrated in cereals, the expression of PR proteins has clearly been identified in cereal defense responses. The study of PR protein expression in the Hobbit 'sib' mutants may give an indication of the effect of mutation on defense response. It might be expected that some mutations will lead to elevated or constitutive expression of PR proteins.

A study of defense gene expression in relation to time after inoculation may also prove informative, indicating at which point in the infection process the resistance takes effect in the mutant. The expression of four defense related genes has been studied in barley (Boyd *et al*, 1994). After inoculation with powdery mildew, the expression of Chitinase, Peroxidase, PAL and a PR-R homologue were measured in terms of RNA expression. The expression of certain genes was shown to be related to certain stages in the infection process. A similar study in the Hobbit

'sib' mutants may indicate the nature and timing of expression of these genes. Microscopic work could also be used to establish the timing and prevention of pathogen development.

7.6 What purpose do genes for susceptibility serve?

It has been demonstrated in a number of wheat cultivars that there are factors present that increase susceptibility of that cultivar to disease. It was discussed in Chapter 1 whether these factors were genes for susceptibility or suppressors of resistance. If they are genes for susceptibility, the effect is a one step process, where the gene directly confers susceptibility. If the factors are suppressors of resistance, this involves a two step process, involving a gene for resistance and a factor that suppresses its expression. It is quite feasible that both explanations may be correct in different examples.

One must therefore ask why these factors exist, and what their identity is. If they are genes for susceptibility, what possible function can they serve? Why have they not been selected against? Genes for susceptibility may code for a basic structural component of the plant that is also required by the pathogen for successful colonisation. These genes may have direct effects on an important physiological process in the plant, and so have been maintained through selection even though increased dosage of these genes may also result in adverse effects due to susceptibility to disease. If these genes do code for structural components, mutation of the gene for susceptibility may instigate a structural change making pathogen attack more difficult, although, of course, this may have an adverse effect on the plant if they are of key importance to the plant.

If the factors conferring susceptibility are suppressors of resistance, it is possible that they function as regulatory factors, preventing over-expression of resistance genes. For example, the *cpr1* mutant in *Arabidopsis* (Bowling *et al*, 1994) exhibits constitutive expression of PR proteins,

and is resistant to *Peronospora parasitica* and *Pseudomonas syringae*. It is possible that such expression would put the plant at a selective disadvantage in a low disease pressure environment, hence the need for regulation. Alternatively, it may be that genes for susceptibility are involved in signal transduction events, and have an influence on resistance expression. For example, in barley, the loci *Rar1* and *Rar2* have been shown to be required for *Mla₁₂*-specified resistance to powdery mildew (Freidenhoven et al., 1994). Similarly, in tomato, two loci named *Rcr-1* and *Rcr-2* have been identified that are required for *Cf-9* mediated resistance to *Cladosporium fulvum*. Genes for susceptibility may affect expression of these genes and thus increase susceptibility.

Alternatively, it may be that the primary role of these factors is not suppression of resistance, but some other function. Suppression may be a secondary or pleiotropic effect, possibly as a result of inter or intra-genomic competition. Awareness of effects similar to this hypothesis has increased significantly in recent years, mostly as a result of the observation of gene silencing in transgenic plants. This is known as 'homology-dependent gene silencing' (Matzke & Matzke, 1995). Various types of experiments with transgenic plants led to the conclusion that increasing the copy number of particular sequences reduced gene expression (review in Finnegan & McElroy, 1994). First, there was often correlation between inserts of multiple linked copies of transgenes and poor gene expression. Secondly, where the same promoter was used to drive two different constructs, introduction of the second construct resulted in inactivation and methylation of the first construct. Thirdly, introduction of sense constructs as controls in antisense experiments resulted in similar levels of silencing in endogenous genes. Fourthly, attempts at over expression of endogenous genes by the introduction of constructs with strong promoters often led to a reduction in expression of both the transgene and the endogenous gene.

Examples of homology-dependent gene silencing can be grouped into two categories: co-suppression, where both transgene and endogenous genes are silenced; trans-inactivation, where expression of one transgene is suppressed by introduction of another. Two mechanisms have been proposed to account for silencing. Silencing may occur at the post-transcriptional level. Work with plant viruses has suggested that elevated levels of particular RNA molecules may trigger RNA degradation (Smith *et al*, 1994). This could explain co-suppression, where RNA degradation of both the transgene and endogenous gene RNA occurs when RNA transcription reaches a certain level. Silencing may also occur by direct physical association of alleles (Meyer *et al*, 1993) or homologous unlinked sequences (Neuhaber *et al*, 1994). Pairing may generate inactive genetic states. It is thought that these mechanisms may have evolved to silence the many copies of transposable elements in plants (Flavell, 1994), but it is feasible that several single copy genes with close sequence homology may also be affected by these mechanisms.

It is possible that the susceptibility identified in certain wheat cultivars is due to homologous silencing of one resistance gene by another. The suppression of one resistance gene by another has been demonstrated in oats. The crown rust resistance gene Pc94 is suppressed or silenced by Pc68 (Chong & Aung, 1996). It has been proposed that the combination of genomes that occurs, either through amphiploidy or breeding leads to intergenomic competition or silencing (T. Aung, pers. comm.). The formation of hexaploid wheat could effectively be viewed as the same process as transformation in terms of genome interaction and the combination of like sequences. A high level of homology has been demonstrated amongst the cereal genomes (Moore *et al*, 1993b). Therefore, as with transformation, the combination of genomes through amphiploidy could effectively bring homologous sequences together. Thus there is potential for gene silencing. Whether RNA mediated, or via physical association, both give possible

explanations for silencing all the resistance genes involved, resulting in susceptible phenotypes.

With further work, the identification of deletions may enable sequencing of genes for susceptibility. If it were demonstrated that there was good sequence homology with known resistance genes, one might conclude that some form of homology dependent gene silencing was occurring in susceptible genotypes. Bearing in mind some of the problems introducing mutation to remove these genes has caused, it may prove more productive to use transformation to alter the expression of these genes, possibly by achieving homology dependent gene silencing of the genes for susceptibility themselves!

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